Artificially Engineered and WRN Protein Bridged DNA Polymerase Lambda Leads to Improved Translesion Synthesis

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The whole of science is nothing more than a refinement of every day thinking.

By Albert Einstein

ABSTRACT

DNA Polymerase (pol) λ is an X family protein sharing 32% amino acid identity to DNA pol β. Structural subdomains of DNA pol λ are similar to DNA pol β namely fingers, palm, thumb and the 8-kDa 5′-deoxyribose phosphate lyase (dRP lyase) domain. DNA pol λ has no proof reading ability since it lacks 3′→5′exonuclease activity and is known to have high affinity for deoxyribonucleosidetriphosphates (dNTPs). DNA Pol λ contains all the critical residues involved in DNA binding, nucleotide binding, nucleotide selection and catalysis of DNA polymerization. On the basis of the biochemical activity DNA pol λ is implicated in translesion DNA synthesis (TLS), base excision repair (BER) and non-homologous end joining (NHEJ). The most common lesion produced by reactive oxygen species is 7,8-dihydro-8-oxoguanine (8-oxo-G). This highly mutagenic lesion leads to G>T transversions as DNA pol λ can either incorporate dATP or dCTP opposite an 8-oxo-G lesion. DNA pol λ is very efficient in performing error-free TLS past an 8-oxo-G along with auxiliary proteins proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A). The auxiliary proteins PCNA and RP-A act as molecular switches to coordinate the pol selection in 8-oxo-G repair.

The aim of the thesis part 1 was to understand the role of DNA pol λ as a translesion repair enzyme over the O-6-methylguanine (O-6-mG) lesion along with auxiliary proteins PCNA and RP-A. In order to understand the role of Tyr505, the highly conserved catalytic residue over O-6-mG, we performed a single hydrophobic amino acid substitution. The data obtained lead to the conclusion that the mutation allows for an error free TLS over O-6-mG. This work contributes to the understanding that a small change in the local environment, can lead to alteration in the hydrogen-bonding pattern, thus remarkably altering the pol fidelity.

In part 2 the role of the functional interactions between the Werner syndrome protein (WRN) and DNA pol λ was investigated. Aging is associated with damage accumulation produced by reactive oxygen species (ROS) in the genome and increased cancer incidence. 8-oxo-G being the most commonly produced lesion by ROS is repaired by DNA pol λ via

the BER pathway. WRN is a RECQ family of DNA helicase and is also known to be associated with an inherited diseases paving way to premature aging, increased cancer incidence and genomic instability. A specific recruitment of WRN and DNA pol λ from whole cell extracts to the A:8-oxo-G DNA template was identified *in vitro* and *in vivo* data demonstrate a functional interaction between DNA pol λ and WRN. The interacting interface of DNA pol λ was identified in its pol β like core domain. Cells exposed to oxidative stress enhanced the WRN and DNA pol λ association with a strong nuclear co-localization signal. These results present strong evidence for the involvement of WRN in oxidative DNA damage repair.

ZUSAMMENFASSUNG

DNA Polymerase (Pol) λ ist ein Protein der X Familie, welches 32% Sequenzidentität mit DNA Pol β aufweist. Strukturelle Subdomänen von DNA Pol λ haben Ähnlichkeit zu den Finger-, Handfläche-, Daumen- und der 8 kDa 5'-Deoxyribosephosphat Lyase (dRP Lyase) Domänen von DNA Pol β. DNA Pol λ hat keine Korrekturfähigkeit, weil ihr die 3'->5' Exonukleaseaktivität fehlt, und sie zeigt hohe Affinität zu Desoxyribonucleosidtriphoshaten (dNTPs). Sie enthält alle notwendigen Reste, welche an der Bindung und Selektion von DNA und Nukleotiden und an der Katalyse der DNA Polymerisierung beteiligt sind. Basierend auf biochemischen Experimenten wird eine Rolle von DNA Pol λ in der Transläsions-DNA-Synthese (TLS), Basen- Exzsions Reparatur (BER) und der Verknüpfung von nichthomologen Enden vermutet. Die häufigste Läsion, welche durch reaktive Sauerstoffspezies hervorgerufen wird, ist 7,8-Dihydro-8-oxoguanin (8-oxo-G). Diese höchst mutagene Läsion führt zu G>T Transversionen, weil DNA Pol λ sowohl dATP als auch dCTP im DNA Strang gegenüber der 8-oxo-G Läsion einfügen kann. Zusammen mit den Hilfsproteinen "Proliferating Cell Nuclear Antigen" (PCNA) und "Replication Protein" (RP-A) kann DNA Pol λ sehr effizient fehlerfreie TLS ausführen. Dies geschieht meist durch BER. Die beiden Hilfsproteine fungieren dabei als molekulare Schalter, welche die Selektion der DNA pol in der 8-oxo-G Reparatur koordinieren.

Das Ziel des ersten Teils dieser Doktorarbeit war die Funktion von DNA Pol λ als Reparaturenzym für die O-6-Methylguanin (O-6-mG) Läsion in Kooperation mit PCNA und RP-A zu untersuchen. Um die Rolle des höchst konservierten katalytischen Rests Tyr505 zu ergründen, wuvde wir eine einzelne hydrophobe Aminosäuresubstitution von Tyr505Met im aktiven Zentrum durchgeführt. Unsere Beobachtungen haben zum Schluss geführt, dass diese Mutation eine fehlerfreie TLS von O-6-mG erlaubt. Damit trägt diese Arbeit zur Sicht bei, dass eine kleine Veränderung, welche das Wasserstoffbrückenmuster verändert, die DNA Pol genanigkeit bemerkenswert beeinflussen kann.

Im zweiten Teil wurde wir die Rolle der funktionalen Interaktion zwischen dem Werner-Syndrom Protein (WRN) und DNA Pol λ untersucht. Das Altern ist mit einer Ansammlung

von Beschädigungen des Genoms durch reaktive Sauerstoffspezies (ROS) und einem erhöhten Auftreten von Krebserkrankungen verbunden. 8-oxo-G ist die häufigste durch ROS hervorgerufene Läsion und wird via BER von DNA Pol λ repariert. WRN ist ein Protein der RecQ Familie von DNA Helicasen und wird assoziiert mit Erbkrankheiten, welche vorzeitiges Altern, erhöhte Krebshäufigkeit und Genominstabilität hervorrufen. Eine Rekrutierung von WRN und DNA Pol λ aus Zellextrakten ans A:8-oxo-G DNA Templat konnte nachgewiesen werden. *In vitro* und *in vivo* Daten zeigen eine funktionale Interaktion zwischen DNA Pol λ und WRN. Als interagierende Berührungsfläche von DNA Pol λ wurde ihre Kerndomäne identifiziert, welche Ähnlichkeit mit DNA Pol β aufweist. Zellen welche oxidativem Stress ausgesetzt sind verstärken die Assoziation zwischen WRN und DNA Pol λ, welche dabei eine starke Kolokalisation im Zellkern zeigen. Diese Resultate stellen einen starken Beweis dar für die Einbindung von WRN in der Reparatur von oxidativen Schäden an der DNA.

Part I

AIM

DNA lesions that are constantly produced by exogenous and endogenous chemicals and other agents can result in uncontrolled duplication of cells and therefore leading to cancer. The ability of certain DNA polymerases to bypass such toxic lesions accurately is required to maintain genomic stability. In context to the above scenario the aim of part 1 of this thesis project was to study translesion synthesis by DNA polymerase λ, a member of the X-family DNA polymerase, over the highly toxic O-6-methylguanine lesion. Single hydrophobic substitution mutants of the active site residues were constructed to unveil its possible role in nucleotide selectivity opposite the lesion. This was extended a step forward to understand the role of the auxiliary proteins proliferating cell nuclear antigen (PCNA) and replication protein (RP-A) in the bypass of the O-6-methylguanine lesion.

INTRODUCTION

O-6-methylguanine (O-6-mG) is a minor modification that leads to highly mutagenic DNA adduct (1). Exogenous compounds like N-methyl-N-nitrosourea, methyl methane sulfonate and N-methyl-N′-nitro-nitrosoguanine, endogenous biochemical compounds like S-adenosylmethionine and metabolically nitrosated amines and amides such as betaine and choline can produce O-6-methylguanine (2-4). Compounds like nitrates and nitrites found in food and beverages can form methylating agents in cells (5).

Methylation of DNA

Twelve different sites on DNA bases are attacked, by most methylating agents, including all the exocyclic oxygen and most ring nitrogens. They can also methylate oxygen atoms in the phosphate of the sugar-phosphate backbone, thereby generating methylphosphotriesters (6,7). O-6-mG DNA lesions are induced by SN1 or SN2 mode of nucleophilic substitution reactions. Methylating agents decide the proportion of alkylation that occurs at different sites of the DNA bases. SN1 agents, such as N-methyl-N-nitrosourea and N-methyl-N′-nitro-nitrosoguanine are highly mutagenic because they react more readily with the oxygens in DNA to generate the main mispairing adduct O-6-mG and also other minor adducts (8). DNA polymerases (pols) stall at these lesions and insert the incorrect base thymine at a frequency of more than 90% during replication (9). Due to the mispairing, with thymine during DNA replication GC→AT transition mutations occurs (10).

Repair of O-6-methyl Guanine

The O-6-mG DNA lesion is recognized and repaired by the methyl guanine DNA methyltransferase (MGMT) protein and by DNA-mismatch repair (MMR) pathway.

O-6-methylguanine methyltransferase (MGMT):

The O-6-mG adduct in DNA is removed by an ubiquitous and unique repair protein, O-6-mG DNA methyltransferease (MGMT; DNA-O-6-methylguanine protein-L-cysteine S-

methyltransferase, EC 2.1.1.63) present in most species (11). Unlike other enzymes this protein transfers the methyl group from the lesion in a stoichiometic second–order reaction (12). The methyl lesion is covalently bound to a cysteine residue within the active site of the protein and is therefore called a "suicidal protein" (13) (Figure 1.1).

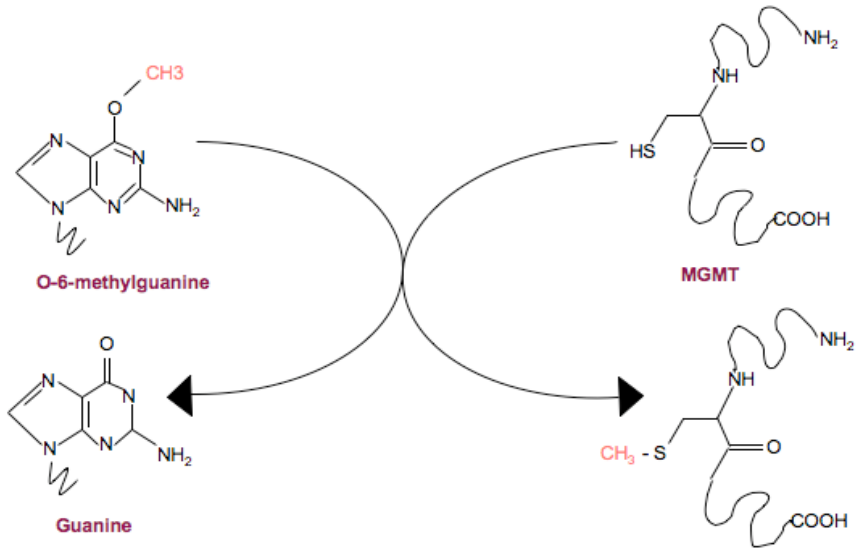

Figure 1: **Reaction mechanism of MGMT**. The O-6-alkylguanine DNA alkyltransferase protein scans double-stranded DNA for alkylation at the O6 position of guanine. Covalent transfer of the alkyl group (in the figure shown as methyl group, CH_3 group) to the conserved active site cysteine inactivates the MGMT protein and restores the guanine to normal. Reproduced from Gerson, S.L, Nature Reviews Cancer, **4**, 943-955 (2004).

In mammals, MGMT expression is highly regulated in different tissues and cell lines (14-16). It functions as an alkyl acceptor of O-6-mG. MGMT can also repair O-6-mG in open chromatin during transcription and replication as well as in condensed DNA. It is known that Mgmt-knockout mice (Mgmt-/-) are more sensitive to methylating agents than the wild-type (wt) mice (Mgmt+/+) counterparts (17,18). This illustrates the importance of MGMT in the repair of O-6-mG lesions.

Mismatch repair (MMR) and O-6-methyl Guanine lesion:

During DNA replication, thymine is mispaired to O-6-mG by replicative DNA pols. If the O-6-mG lesion is not repaired by MGMT a O-6-mG:T mismatch, can result leading to a subsequent mutation on the daughter strand. The O-6-mG:T mispair lesion is then

recognized by the MMR proteins MutSα and MutSβ and hence the MMR repair machinary removes the dT (19). However, the O-6-mG, during the next around of replication will again pair with thymine, allowing for repeated binding of the MMR proteins. Therefore the processing of O-6-mG is aberrant. This leads to apoptosis resulting from several rounds of repair and single-strand breaks. However there are recent evidences for the recruitment of the DNA damage signaling kinase ATM and/or ATR to the site of O-6-mG:T after recognition by the MMR proteins (20). These proteins then directly signal for cell cycle checkpoints and apoptosis. Exonuclease 1 plays a vital role in the MMR pathway and is known to be involved in eliciting apoptosis (21) (Figure 2).

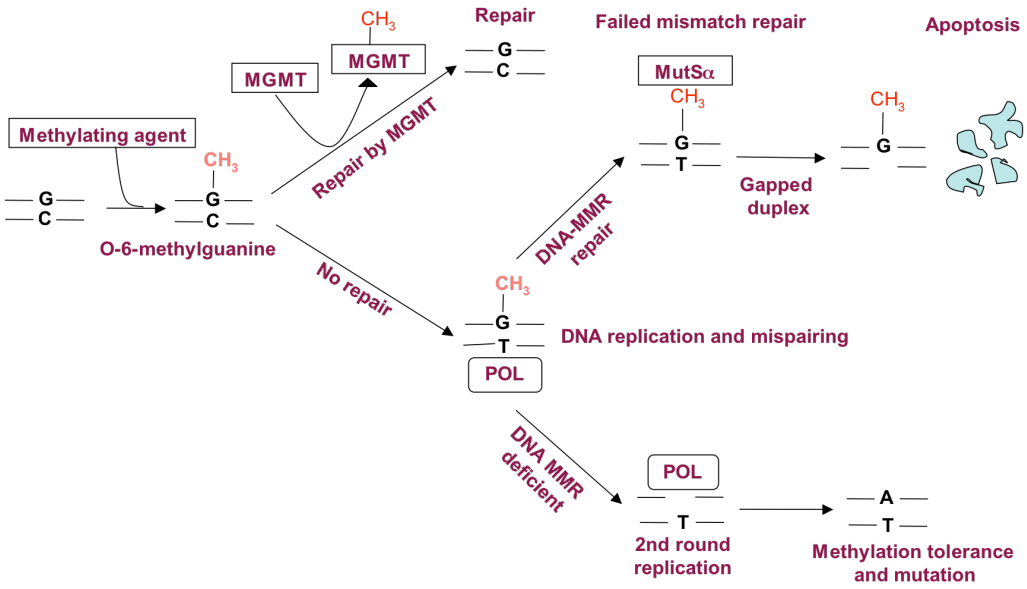

Figure 2. **DNA repair mechanisms of a O-6-mG damage**. After a O-6-mG alterations possible outcomes include: (1) reapir by MGMT. (2) apoptosis (3) methylation tolerence and induction of mutations. The later outcome is predicted to be responsible for transformation. For details see text. Reproduced from J. M. Allan & L. B.Travis, Nature Reviews Cancer, **5**, 943-955 (2005).

DNA polymerases and O-6-methyl Guanine lesions:

Past evidences have proven that O-6-mG lesion blocked the *E.coli* DNA pol I Klenow fragment. Eukaryotic DNA pol α, which is involved in lagging strand DNA synthesis is strongly blocked one base before the lesion, suggesting an inhibition of nucleotide

incorporation opposite the lesion (22,23). The Y family translesion DNA pol ι and κ produce mainly one nucleotide base incorporation, whereas as DNA pol η could bypass O-6-mG (24,25). Steady state kinetic analysis indicated a similar efficiency of insertion opposite the lesion for dCTP and dTTP by DNA pols η and κ. However DNA pol ι showed preferential incorporation for dTTP (26,27). DNA pol ζ is highly inefficient at inserting nucleotide opposite the lesion, but can efficiently extend from the nucleotide inserted opposite the lesion. DNA pol β, together with G:T-specific thymine-DNA glycosylase was shown to be involved in the futile cycling at the O-6-mG lesions along with MutSα (22,25). Processing of O-6-mG by mismatch correction also requires PCNA and DNA pol δ and/or ε (28). The above studies indicated that all known DNA pols incorporate the incorrect thymine at equal or higher frequency than the correct cytosine opposite O-6-mG.

DNA polymerases

Based on sequence homologies and structural similarities, DNA pols are grouped in the seven different families. A, B, C, D, X, Y and reverse transcriptase (RT) (29). The family X of DNA pols contain DNA pol β, λ, μ and terminal transferase (Tdt). X family DNA pols are small proteins found in vertebrates and are known to function in DNA repair (30).

DNA polymerase λ:

Human DNA pol λ is a 68kDa protein encoded by 575 amino acid by the POLL gene and maps to chromosome 10q23. DNA pol λ shares 32% amino acid sequence homology to human DNA pol β (31,32). DNA pol λ is a single subunit protein, which catalyzes DNA synthesis in a template dependent manner and has no associated 3′→5′ exonuclease activity (33). The N-terminal BRCT domain is involved in protein-protein interactions; the pol β like domain is the catalytic core and a serine-proline rich region (34), which is suggested to be a target for posttranslational modification like phosphorylation, known to maintain protein stability (34-36). The catalytic core is composed of an N-terminal 8kDa domain and a polymerase domain including the fingers, palm and the thumb (37) (Figure 3). DNA pol λ

is a template dependent DNA pol, but can perform template independent incorporation with low efficiency (38). Strand displacement activity is limited by the presence of a phosphate at the 5′ end of the gap by DNA pol λ. A high affinity for dNTPs by DNA pol λ, unlike DNA pol β, suggests a possible role in DNA repair when the intra-cellular dNTP concentrations are low (38). The dRP lyase activity of DNA pol λ is likely known to proceed via beta elimination using Lys310 as a major catalytic residue in short patch BER (39,40).

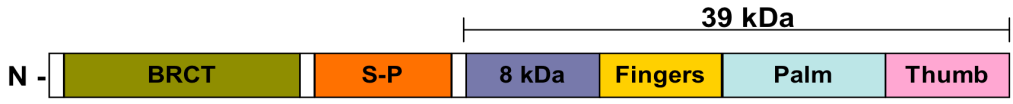

Figure 3. **Domain organization of DNA polymerase λ.** The domain of DNA pol λ site: BRCT, Ser-Pro rich domain (S-P), 8 kDa domain and the polymerase catalytic domain composed of fingers, palm and thumb subdomains. The 39kDa domain is very homologous to DNA pol β. Reproduced from Garcia-Diaz, M. et. al., DNA Repair, **4,** 1358-1367 (2005).

Functional roles of DNA polymerase λ:

DNA pol λ shares similar biochemical activity as DNA pol β including the dRP lyase activity, hence a role in BER was proposed (41,42). DNA pol λ is able to perform TLS in an error free manner over 2-hydroxy-adenine and a more often formed lesion produced by reactive oxygen species called 7,8-dihydroxoguanine (8-oxo-G) along with auxiliary proteins PCNA and RP-A (43). In a very recent study DNA pol λ along with MUTYH was shown to have a role in DNA repair of 8-oxo-G lesion (44). DNA pol λ also functions in non-homologous end joining (NHEJ). The BRCT domain of pol λ is important for interaction with proteins that function in NHEJ (45,46). DNA pol λ is also known to play a role in V(D)J recombination by joining of the heavy chain junctions in B-cells since mice deleted of DNA pol λ have shorter junctions (47).

Structural insight in DNA polymerase λ

DNA Pol λ fills in short gaps when the 5′ end of the gap contains a 5′-phosphate. This phenomenon is explained by the presence of an 8kDa domain that is known to interact with the 5`-phosphate (48) (Figure 4).

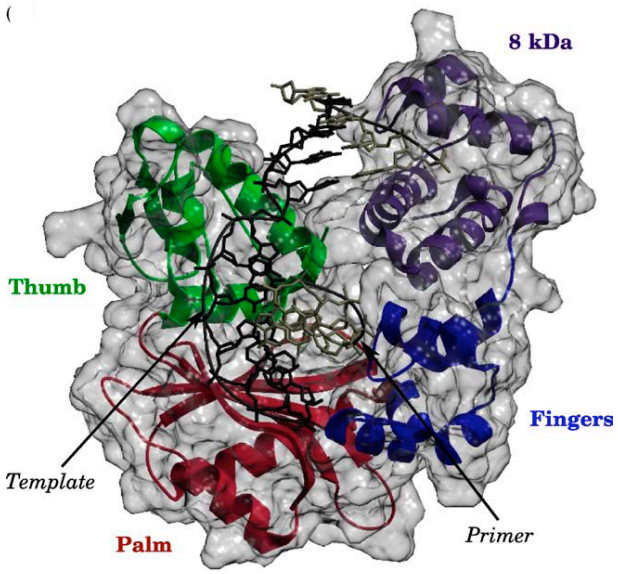

Figure 4: **Subdomain organization of DNA polymerase λ.** Ribbon representation of the 39kDa catalytic core of DNA pol λ in complex with a two-nucleotide gap (the DNA is shown in stick representation). The molecular surface is shown in transparent gray. The atomic coordinate corresponds to PBD IRZT. Reproduced from Garcia-Diaz, M. et. al., DNA Repair, **4,** 1358-1367 (2005).

Analyses of the X-ray crystal and computational studies of binary state (DNA pol λ complexed with DNA) and ternary state (DNA pol λ bound to the DNA and the correct incoming dNTP) complexes have shown that DNA pol λ undergoes a dNTP induced conformational change. It is known that the conformational change form a binary to the ternary state of DNA pol λ is minor in comparison to DNA pol β, another DNA pol from the same X family. DNA pol λ remains in a close subdomain conformation whether or not an incoming nucleotide is bound. Although, upon binding of the correct incoming nucleotide, the enzyme:DNA complex undergoes a transition form an inactive state (closed state) to an active state (open state) (49-51). The active site amino acid residues help to coordinate the chemical reaction of the enzyme:DNA complex. These protein residues act as the steric gatekeepers regulating the assembly of the active site of the chemistry (52).

Tyr505 and Phe506 of DNA pol λ are important for nucleotide discrimination. Tyr505 moves in order to permit entry of the dNTP into the active site of DNA pol λ (48,51). This movement allows for the repositioning of DNA templating base into the active site. Phe506 blocks interaction between Arg258 and Asp192 in closed state allowing for DNA motion towards the closed conformation. IIe492 interacts with Asp192 and sterically inhibits the flipping of the Phe506 to a closed conformation. Arg517 is important for coupling of the DNA template movement to the binding of the correct dNTP in the closed state (50). Finally it has been shown that DNA pol λ is able to perform gap filling longer than 1nt during DNA repair and this was structurally explained by a mechanism called "scrunching" (53).

Proliferating Cell Nuclear Antigen (PCNA)

PCNA is a protein that is evolutionarily conserved and is found in all eukaryotic species from yeast to human as well as in archea and a member of the DNA sliding clamp proteins. In *E.coli* the DNA pol III β-subunit and in *E.coli* bacteriophage T4, the gene 45 protein regulate the processivity of the replicative DNA pols (54,55). PCNA is functionally associated with DNA replication, DNA repair, chromatin remodeling, cell cycle control and sister-chromatid cohesion.

Structural insight:

Alignment of amino acid sequences of PCNA from different species show considerable homology and evolutionary conservation. Surprisingly the yeast and human PCNA share only 35% sequence homology, but their three-dimensional structure is highly superimposable (56). The eukaryotic PCNA is composed of three identical monomers, linked in a head to tail fashion, forming a homotrimer and an overall toroidal shape. Each monomer consists of two similar domains linked by an interdomain connecting loop (57-59). There are two PCNA-specific binding motifs identified, of which the PIP-box (PCNA-interacting protein box) is the best studied so far (60,61). The consensus sequence of the PIP box is QXX (M/L/I) XX (F/Y)(F/Y). Another PCNA binding motif (KA

(A/L/I)(A/L/Q) XX (L/V) known as KA-box was identified which is distinct from the classical PIP-Box (62). Residues L121 to E132 at the interdomain connecting loop of PCNA was mapped to be the interacting sites for many proteins. This loop is recognized by several proteins like DNA pol δ, p21, flap-endonuclease 1 (FEN-1) and DNA ligase I (63-66). Another protein interacting sequences is in the N-terminal region including the inner α-helices, which forms part of the binding site for cyclin D and the C-terminal tail, for the interaction with DNA pol ε, replication factor C (RF-C), CDK2 and GADD45 (67-69).

Post-translational modifications of PCNA:

PCNA is modified by different post-translational mechanism like ubiquitination, phosphorylation, acetylation, methylation and sumoylation. One of the well-documented PCNA posttranslational modifications is ubiqutination. Lysine residues at position 164 are the highly conserved and targeted for ubiquitination. Attachment of a single ubiquitin moiety leads to monoubiquitination and building up of ubiquitin chain through Lys^{63} results in polyubiquitination (70). Monoubiqutination occurs in a sequential manner, ubiquitin-activating enzyme E1 being the first, the ubiquitin-conjugating enzyme E2 (which in humans might be either Rad6A or Rad6B) and a RING finger containing E3 ubiquitin ligase called Rad18 (71,72). Polyubiquitin chain building requires the ubiquitin-conjugating enzyme Ubcl13-Mms2 and a specific RING-finger containing E3 ubiqutin ligase (73). In yeast it is Rad 5 and in humans SHPRH (SNF2 histone, linker PHD RING helicase), HTLF (helicase like transcription factor) and RNF8 (ring finger protein 8) (74-76). Lysine 164 of PCNA is also the target for another modification designated as sumoylation (70). It involves the attachment of a small protein called SUMO (small ubiquitin related modifier) observed in yeast. Lysine 127 is also a known target for sumoylation, lacking in higher eukaryotes. Sumoylation of yeast PCNA, is a trigger for recruitment of a specific DNA helicase (ySrs2), which blocks recombination events during replication (77).

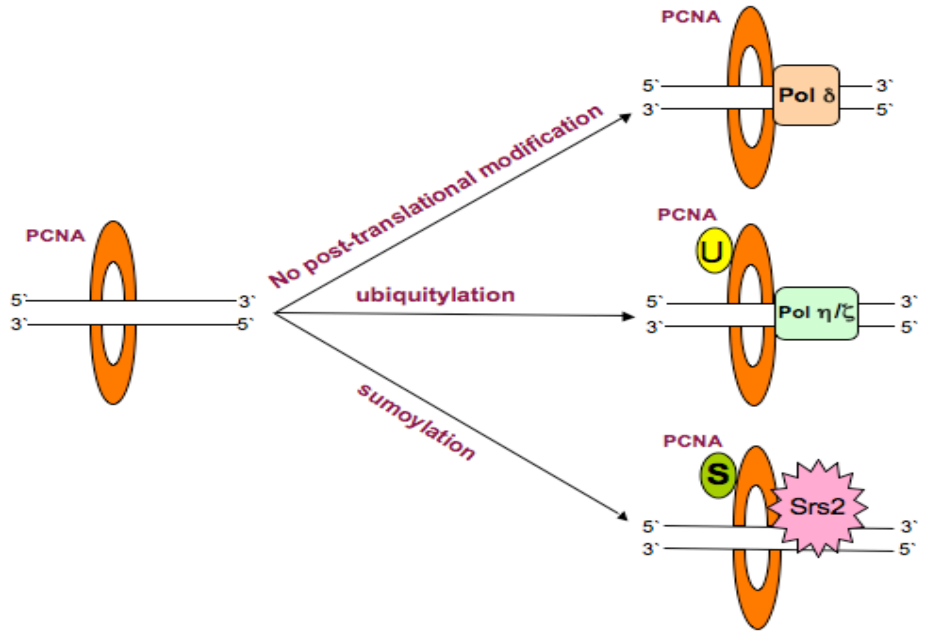

Figure 5.**Ubiqutination and sumoylation of PCNA:** Ubiquitylation (U) or sumoylation (S) of PCNA leads to affinity changes for DNA pol and in the case of sumoylation, the recruitment of helicase Srs2. Reproduced from Lopez de Saro, Current Genomics, **10**, 206-215 (2009).

Phosphorylation of Tyr21 in both humans and mouse cells stabilizes chromatin bound PCNA (78). Acetylation is another modification detected on PCNA. Acetylated and hyperacetalyted PCNA are found on the chromatin and localization of the non-acetylated form in the nucleosol, strongly suggest that PCNA translocation is dependent on the acetylating status (79). Methyl esterification of PCNA on glutamic acid and aspartic acid is a new modification and its functional consequence have yet to be unveiled in detail (80).

Role of PCNA in DNA repair and translesion synthesis:

PCNA is an indispensable part of several repair pathways such as mismatch repair (MMR), nucleotide excision repair (NER) and BER (54,55). PCNA interacts with MutS homologue1 (MSH3), MutS homologue (MSH6), exonuclease 1 (Exo1) and MutL homologue (MLH1), components of the MMR pathway (81,82). The endonuclease xeroderma pigmentosum complementation group G (XPG) known to play a role in NER pathway interacts with PCNA (83). DNA damage caused by oxidating, reducing or alkylating agents and misincorporated uracils is repaired by BER. PCNA is associated with

the recruitment of BER proteins to the site of action via long-patch BER. Its interacting patners are DNA glycosylase (uracil DNA glycosylase, UNG2), methylpurine-DNA glycosylase (MPG), NTHL1 (endonuclease III), human MutY homologue (hMYH), AP-endonuclases, DNA pols δ, β and ε and X-ray repair complementing defective repair in Chinese-hamster cells (XRCC1) (reviewed in (84)).

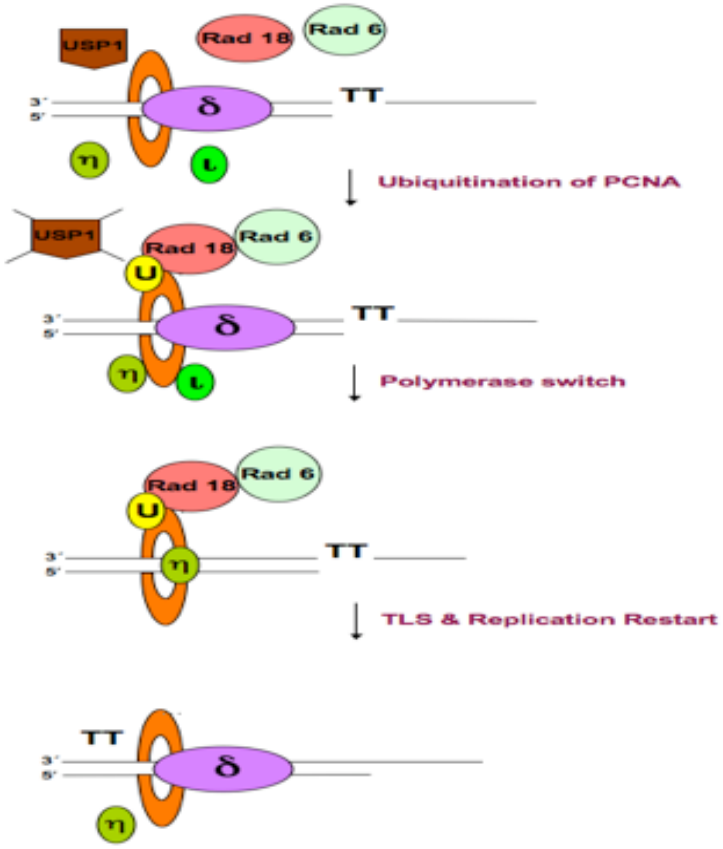

Figure 6.**Translesion DNA synthesis and DNA polymerase switch.** The example given demonstrates TLS and DNA pol switch after thymine dimers. Courtesy to Ursula Hübscher.

In eukaryotic cells a crucial pathway for DNA damage tolerance is translesion synthesis (TLS). There are specific DNA pols that are able to carry out DNA synthesis past different lesions. Recent results have shown that the lesion bypass activites of several TLS DNA pols are increased by their physical interaction with PCNA. Since TLS requires the

interplay between the specialized DNA pols η, ι, κ, λ and the replicative DNA pol δ and ε (85). PCNA, with its different posttranslational modifications is an ideal candidate for coordinating their functions and recruiting them to the replication fork after the damage has occurred. The switch from a replicative DNA pol δ to TLS DNA pol η and ζ is determined by the monoubiqutination of PCNA (86) (Figure5). Figure 6 shows a model for TLS and the DNA pol switch after DNA damage.

Replication protein A (RP-A)

RP-A is a human single strand DNA (ssDNA) binding protein (87). ssDNA is an intermediate of DNA metabolism which has to be protected from unwanted attack by endonucleases and genotoxic agents (88). RP-A is localized in the nucleus and is present in the replication foci in human cells (89-91).

Structure of RP-A and binding mode to single-stranded DNA:

In eukaryotes RP-A is a heterotrimer composed of three tightly associated subunits of 70, 32 and 14 kDa (refered to as RPA-70, RPA-32 and RPA-14, respectively). RP-A binds ssDNA in a highly sequential manner with a 5` to 3` binding polarity (92-94). The oligosaccharide / oligonucleotides binding fold (OB-fold) is the central structural and functional element of RP-A. RP-A is known to contains six OB folds, each of which consists of five β-strands arranged in a β-barrel shape. The RP-A 70 subunit contains four OB-folds designated as DNA binding domain A (DBD-A), DBD-B, DBD-C, and DBD-F. RP-A 32 subunit contains DBD-D and RP-A 14 has DBD-E. DBD-A and DBD-B of RP-A 70 is the major ss DNA binding domain. DBD-A and DBD-B initiate the binding interaction with a length of 8-10 nucleotides at the 5`-side of ssDNA. The binding of 13-22 nucleotide occurs with the additional involvement of DBD-C. Hence a cooperative binding of all four RP-A DBD (A-D) requires approx 30nt of ssDNA (95,96). In addition to the above-mentioned domain RP-A 32 also contains an unsaturated N-terminal phosphorylation domain. RP-A is known to be phosphorylated in a cell cycle dependent manner. RP-A undergoes phosphorylation during G1/S transition and in the M-phase

(97,98). Thus the functional role of RP-A hyperphosphorylation might be to shift a fraction of the cellular pool of RP-A from replication to DNA repair (99).

Interaction of RP-A with DNA repair proteins:

RP-A is required for all major repair pathways namely NER, BER, MMR and DNA double-strand break repair (DSBR) and homologous recombination (HR). In NER RP-A is involved in recruiting XPG, XPA and ERCCI-XPF endonuclease (100-102). RP-A participates in the gap filling reaction along with PCNA and DNA pol δ and ε. RP-A was implicated in BER via interaction with human uracil-DNA glycosylase (UNG2) and its stimulatory effect on long-patch BER. RP-A is also involved in MMR repair. RP-A has also been shown to interact with RAD52 and RAD51 proteins in the homologous recombination pathway (103). Human RP-A can also interact with the breast cancer susceptibility proteins BRCA1 and BRCA2 as well as tumor suppressor p53 protein (104,105).

PAPER I

Hydrophobic substitution mutation of the Y505 residue of human DNA polymerase λ increases fidelity and reduces translesion synthesis over the O-6-methylguanine lesion.

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Running title: Bypass of O-6-mG by DNA polymerase λ.

Abstract

DNA polymerase λ (pol) a member of the DNA polymerase X family has a role in non-homologous end joining and is believed to play an important role as an accurate translesion DNA pol in base excision repair after DNA replication. We show here that a hydrophobic substitution mutant of the highly conserved residue at the active site of human DNA pol λ (Y505M) allows better incorporation of the correct nucleotide (dCTP) over an O-6-methylguanine (O-6-mG) template relative to the non-complementary nucleotide (dTTP) usually incorporated by a variety of DNA pols. In contrast the wild-type DNA pol λ incorporated as expected preferentially the incorrect dTTP. This is the first report of a DNA pol showing faithful bypass of the O-6-mG lesion and can help to elucidate the molecular basis for the mutagenic potential of this lesion. When, however, RP-A was included in the reaction on the O-6-mG lesion inhibition of dCTP and dTTP incorporation was seen for both the wildtype and the Y505M mutant DNA pol λ suggesting a modulatory role of this auxiliary protein over this lesion.

Introduction

There is substantial evidence that genetic instability is displayed with increased rate of errors in DNA synthesis. In this process, nucleotide selection within the active site of a DNA polymerase (pol) is the major contribution to the fidelity of DNA synthesis (1). Nevertheless, numerous endogenous and exogenous agents damage DNA. Some of the damage may escape repair and compromise the fidelity of replication and transcription (2). One such DNA lesion is O-6-mG, caused by exogenous methylating agents like N-methyl-N-nitrosourea, methyl methane sulfonate, N-methyl-N′-nitro-N-nitrosoguanidine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone produced by tobacco burning and endogenous products of metabolism like betaine and choline, along with chemotherapeutic agents procarbazine and temazolomide (3-5). Methylating agents primarily react with exocyclic nitrogen or oxygen atoms by adding a methyl group on purines and pyrimidines, resulting in an impaired adduct, O-6-mG (2). The adduct O-6-mG mispairs with an incorrect thymine during DNA replication instead of a correct cytosine, resulting in GC→AT transition mutation (6). A ubiquitous O-6-methylguanine-DNA-methyltransferase (MGMT) in a stoichiometric fashion repairs O-6-mG by directly reverting DNA base damage (7). MGMT transfers the methyl group from the mutagenic DNA lesion to a cysteine residue with in the active site, rendering it incapable of continued repair, hence the number of lesion that can the cleared from a cells genome is limited by the available MGMT molecules, unless more of the protein is made (8). This type of repair is error free and noncytotoxic. However, in many human solid tumor cell lines and in some non-tumor tissues the ability to repair O-6-mG is lacking due to the inactivation of MGMT (9). The initial event in O-6-mG induced mismatch repair may be the recognition of dT-O-6-mG (after removal of methyl group by MGMT) by the hMSH2. Functional deficiency of these mismatch recognition proteins imparts resistance to alkylation-induced cytotoxicity (10,11). Alternatively, a G:T specific thymine DNA glycosylase can also remove thymine from dT-O-6-mG base pair, allowing for the abasic site to be acted upon by apurinic/apyrimidinic (AP) endonucleases. However, it was shown that DNA pol β along with G:T-specific thymine-DNA glycosylase (along with other proteins such as MutSα) may be involved in the futile cycling at the O-6-mG lesions (9).

It has been shown that O-6-mG lesion blocked *E.coli* DNA pol I Klenow fragment as well as the eukaryotic DNA pol α involved in DNA replication (9,12). The translesion synthesis (TLS) Y family DNA pols η and κ show similar efficiencies for insertion of dCTP and dTTP opposite the O-6-mG, while DNA pol ι, which had a strong preference for dTTP, along with the major repair enzyme DNA pol β (13,14). Another report suggested that DNA pol κ is a poor, TLS DNA pol over O-6-mG, but when it incorporated either T or C efficient elongation is achieved by the replicative DNA pol δ (15). The above studies indicated that all known DNA pols incorporate the incorrect thymine at equal or higher frequency than the correct cytosine opposite O-6-mG. DNA pol λ was shown to incorporate dTTP at relatively high efficiency then dCTP for TLS past O-6-mG (16). Recently we have shown that pol λ is very efficient in performing error-free TLS past an 8-oxo-G and 2-OH-A lesions along with auxiliary proteins PCNA and RP-A (17,18).

In order to understand the carcinogenic mechanism of O-6-mG we studied the behaviour of DNA pol λ in the presence of the auxiliary proteins RP-A and PCNA. We furthermore constructed and characterized the biochemical properties of DNA pol λ wt by exchanging the conserved Y505 amino acid of the nucleotide-binding domain with hydrophobic amino acid residue methionine in the presence of auxiliary proteins RP-A and PCNA. DNA Pol λ conserved active site residue Y505 was shown to be involved in determining nucleotide selectivity opposite the lesion (19-21). Work of the group of Marx et al suggesting that hydrophobic substitution mutations can result in a more selective enzyme (22,23). Our data suggests that Y505M mutant is a novel in-vitro evolved DNA pol λ that allows the error free TLS over O-6-mG by incorporating the correct dCTP. This is in contrast to the parental pol λ wt, as expected, incorporated the wrong nucleotide dTTP. Finally, the auxiliary protein RP-A displays a curial role in negatively regulating the TLS of both pol λ wt and Y505M over O-6-mG.

Materials and Methods

Oligonucleotide synthesis

Oligonucleotides were synthesized by Purimex. All oligonucleotides were purified by denaturing polyacrylamide gels (PAGE).

Enzymes and Proteins

The bacterial expression plasmid pRSET-B encoding human DNA pol λ wt and the Tyr505Ala were cloned, expressed and purified as described previously (24). Tyr505Meth was cloned and purified as described previously, Wimmer et al (25). Recombinant human PCNA and human RP-A were express and purified as described, Maga et al (17). After purification, the proteins were >90% homogenous as judged by SDS-PAGE and Coomassie staining.

Single nucleotide insertion assays

The primer/template complexes (whose sequence as indicated in the respective Figure) were annealed as described in the primer extension assay. The reactions were initiated by adding 10μM of the indicated dNTP (unless indicated in the figures), in a reaction mixture (10μl) containing 50mM Tris-HCl pH 7.0, 0.25mg/ml BSA, 10mM DTT and 10fmol of the 5′^{32}P-labelled primer template, DNA pol λ wt, Y505M, and Y505A at 40nM and 1.0mM Mg^{2+}, and incubated at 37°C for 15min. After incubation, reactions were stopped by adding loading buffer (95% formamide, 10mM EDTA, Xylene cyanol and Bromophenol blue) heated at 95°C for 3min, loaded and separated on a 7M Urea 15% polyacrylamide gel and the products mixture analysed by autoradiography.

Sequence dependent incorporation assay

The primer/template complexes were annealed as described in the primer extension assays (sequence as depicted in the respective Figure). In order to determine the rates of dCTP and

dTTP incorporation with respect to nucleotide substitution after the lesion, dCTP and dTTP were titrated from 1 to 100μM in reaction conditions as stated above in the primer extension assay. The observed rates of nucleotide incorporation were calculated from the values of integrated gel band intensities.

I^*_T/I_T-1

Where T is the target site, the template position of interest; I^*_T is the sum of the integrated intensities at positions T, T + 1,……, T + n

All the intensity values were normalized to the total intensity of the corresponding lane to correct for difference in gel loading. The apparent K_m and k_{cat} values were calculated by plotting the initial velocities in dependence of the nucleotide concentration [dCTP] and [dTTP], and fitting the data according to the Michaelis –Menten equation:

K_{cat} $[E]_o/(I+k_m/[dNTP])$

Where $[E]_0$, was the input enzyme concentrations. Nucleotide concentrations used were 1μM, 10μM and 100μM. Nucleotide incorporation efficiencies were defined as the k_{cat}/K_m ratio. Under single nucleotide incorporation conditions $k_{cat}=k_{pol}k_{off}/(k_{pol}+k_{off})$ and $K_m=K_sk_{off}/(k_{pol}+k_{off})$, where k_{pol} is the true polymerization rate, koff is the constant for nucleotide binding. Thus, k_{cat}/K_m values are equal to k_{pol}/K_s.

Results

The error-prone bypass of O-6-mG by wild-type DNA polymerase λ is negatively regulated by RP-A.

DNA pol λ wt was first were analysed on 39:72 template, containing a single O-6-mG lesion, in the presence of the indicated dNTP. DNA pol λ wt incorporated the "correct" nucleotide" (dCTP) opposite normal G in contrast allowing for "incorrect nucleotide" (dTTP) incorporation opposite O-6-mG compared to the dCTP insertion (Fig 1a lane 3 and Fig 1b lanes 3 and 5). We have previously shown that the auxiliary proteins PCNA and RP-A are able to increase the catalytic efficiency, fidelity and TLS of DNA pol λ over 8-oxo-G and 2-OH-A. As shown in Fig 1c (lanes 1-4, 6-9) increasing concentrations of RP-A alone was able to inhibit dCTP and dTTP incorporation opposite the O-6-mG by DNA pol λ wt

in a concentration dependent manner. However dCTP incorporation by DNA pol λ wt was reduced by 79% and the dTTP incorporation was reduced by 66% by RP-A. PCNA showed no effect on dCTP and dTTP incorporation opposite O-6-mG (Fig 1d, lanes 1-4 and lanes 6-9). These data demonstrate that RP-A has a curial role in negatively regulating the error prone TLS by DNA pol λ wt across O-6mG.

Single base substitution of DNA polymerase Y505M allows a reduced DNA polymerization.

We have previously shown that a highly conserved (Fig 2a) DNA pol λ residue Tyr505 is important for nucleotide discrimination, being involved in interactions with the incoming dNTP. We therefore tested the mutant Y505M and the wt for DNA polymerase activity a 5` -end-labelled 39:72 primer/template DNA oligonucleotide substrate. All the reactions were performed in the presence of Mg^{2+} and the products were resolved by sequencing gel analysis. As shown in (Fig 2b) the Y505M mutant displayed a reduced activity (lanes 7-10) compared to the DNA pol λ wt (lanes 2-5). The kinetic parameters for nucleotide incorporation for both the enzymes were determined as shown in (Fig 2c). However, Y505M substitution showed an approx two-fold decrease in the k_m for the nucleotide substrate with respect to DNA pol λ wt. As a result, the Y505M mutant displayed a two-fold reduction in the nucleotide utilization efficiency (k_{cat}/K_m).

The mutant DNA polymerase λ Y505M but not the wildtype can incorporate the correct dCTP opposite a O-6-mG in a single nucleotide insertion experiment

Both wt and Y505M DNA pol λ were next analysed on 39:72 template, but containing a single O-6-mG lesion, in the presence of the indicated dNTP. Y505M incorporated the "correct" (dCTP) opposite O-6-mG when compared to the dCTP insertion by pol λ wt (Fig 3a, compare lanes 3 and 8). On the opposite the mutant Y505M catalysed "incorrect" dTTP insertion opposite O-6-mG at a highly reduced rate compared to the DNA pol λ wt (lanes 5 and 10). In comparison, no difference was noticed against undamaged dG for both DNA pol λ wt and Y505M (Fig 3b lanes 3, 8). Interesting Y505M showed poor TLS over O-6-mG in the presence of all four nucleotide in comparison to wt (Fig 3a, compare lanes 6 and

11). In order to further investigate these effects, dNTPs titrations were performed in the presence of an enzyme concentration and the data was fitted to Michaelis-Menten equation. As shown in (Fig 3c) the DNA pol λ wt incorporates dTTP opposite O-6-mG almost 3-fold better than dCTP, while Y505M did not incorporate any "incorrect" dTTP opposite the lesion. Moreover, Y505M incorporated the "correct" dCTP opposite the lesion almost 9-fold better than the wt. The kinetic parameters are summarised in Table 1. Neither wt nor Y505M showed any difference in misincorporation of dTTP opposite dG (Fig 3d), suggesting that Y505M does not induce any increase in discrimination against a normal T:G mismatch. However on substituting Y505 with the non-hydrophobic amino acid alanine incorporated the dTTP more preferentially than the correct dCTP (Fig 3e, compare lane 3 and 5).

The Y505M DNA polymerase λ mutant did not have an intrinsic preference for the incorporation of dCTP

When both the wt and Y505M DNA pols were tested in the presence of 8-oxo-G lesion, they both incorporated dCTP better than dATP opposite the lesion, without differences between them, suggesting that the observed effect for the mutant Y505M was specific for dCTP incorporation against O-6-mG (Fig 4a compare lanes 3 and 8). Similarly, when tested on an 8-oxo-A template, both enzymes showed preferential incorporation of dGTP without any difference between wt and Y505M (Fig 4b compare lanes 4 and 9). These two control experiments suggested that the Y505M mutant did not have an intrinsic preference for the incorporation of dCTP.

The Y505M DNA polymerase λ favours dCTP incorporation opposite O-6-mG independently from the sequence context.

To test for the effect of flanking nucleotides on O-6-mG, we next titrated dCTP and dTTP on primer/template where the first nucleotides in the template strand was O-6-mG, followed by either G, A, T or C respectively. No significant difference was observed, with respect to dCTP or dTTP incorporation, on O-6-mG/C and O-6-mG/T templates (Figure S1A). With the O-6-mG/G template, it was seen that the Y505M mutant was more efficient

than wt in incorporating dCTP (Fig 5a) and less efficient in incorporating dTTP (Fig 5c). Similarly, with the template O-6-mG/A the wt was more efficient than Y505M in incorporating dTTP (Fig 3b) but less efficient in incorporating dCTP (Fig 5d). As expected most product accumulated at position +2 (Fig 5a and b). However significant accumulation of +3/4 products could be seen with the O-6-mG/G template and dCTP as substrate (Fig 5a), whereas only very few +3 products were generated with the O-6-mG/A template in combination with dTTP (Fig 5b). This indicated that in the presence of dCTP, DNA pol λ makes template slippage at the C, at +3 position, thus the +3/4 products are generated by using the GG at position 4 and 5 as the templating bases. This explains the fact that only a minimal amount of +3 products are detected with 6-mG/A templates in combination with dTTP, since in such case no slippage can occur.

The error-free bypass of O-6-mG by DNA polymerase λ Y505M is also negatively regulated by RP-A.

The data so far indicated that Y505M was able to perform error-free incorporation of dCTP opposite O-6-mG more efficiently than the wt, at an expense of reduced translesion synthesis. Thus, finally the PCNA and RP-A effects on O-6mG bypass by Y505M was investigated. As shown in Fig 6a (lanes 1-4, 6-9) increasing concentration of RP-A alone was also able to inhibit dCTP and dTTP incorporation opposite the O-6-mG by Y505M in a concentration dependent manner. Lanes 0 and 5 are control reactions in the absence of dNTPs. However the "correct" dCTP incorporation by Y505M was reduced by 53% under the same condition as DNA pol λ wt displaying the fact the Y505M still allows for error-free incorporation opposite O-6-mG under such conditions. The "wrong" dTTP incorporation was reduced by 80% by Y505M respectively (Fig 6b). When tested PCNA showed no effect on dCTP and dTTP incorporation opposite O-6-mG (Fig 6c lanes). These data suggest that RP-A has a curial role in negatively regulating the TLS by pol λ wt and Y505M across O-6mG.

Discussion

DNA encounters various assaults in the native structure and sequence throughout the life span of a cell. The common types of DNA damage like the O-6-mG damage that interferes with replication fork progression can cause cell-cycle arrest and cell death. Furthermore, DNA lesions that persist into the S phase of the cell cycle can obstruct replication fork progression, resulting in the formation of replication-associated DNA double-strand breaks (DSBs) (26). DSBs are generally considered to be the most toxic of all DNA lesion. The fact that all known DNA pols to date show a preferential incorporation of T opposite O-6-mG, led us to investigate the molecular mechanisms for nucleotide selection by DNA pol λ opposite this particular lesion along with auxiliary proteins. RP-A is an important "sensor" protein for DNA replication fork stalling and was shown to act as a molecular switch to activate DNA pol λ dependent highly efficient and faithful repair of 8-oxo-G, repressing DNA pol β activity along with PCNA (27).RP-A inhibited the dCTP and dTTP incorporation by DNA pol λ wt, suggesting that RP-A effect is influenced by DNA template containing lesion. PCNA, which is known to interact both functionally and physically with DNA pol λ showed no effect over this lesion .The above finding is in contrast to the recent data from our laboratories suggesting that DNA pol λ was most accurate in dealing with 8-oxo-G and 2-OH-A in combination with PCNA and RP-A. This allows for strong evidence that RP-A and PCNA plays a vital role in oxidative repair and also allows for MGMT or mismatch repair (MMR) proteins to take over O-6-mG. However the hydrophobic substitution mutation of the active site residue Y505 presented in this study is believed to be involved in dNTP binding completely changed nucleotide incorporation selectivity opposite the O-6-mG lesion by preferentially incorporating the correct nucleotide dCTP. This novel phenomenon was not shared by non-hydrophobic mutation Y505 to alanine and other DNA pols belonging to the X family, which produced high error rates when replicating over the O-6-mG lesion. Our data also shows that the preferential incorporation of dCTP opposite O-6-mG by Y505, is specific for this lesion, since no differences in incorporation opposite 8-oxo-G or 8-oxo-A between the Y505M mutant and the wt enzyme could be seen. Pol λ wt and Y505M misincorporation frequency of deoxynucleotide triphosphate opposite O-6-mG are not influenced by each possible

nearest-neighbour context, contrasts with in vitro primer extension studies showing that misincorporation by other pols is dependent on the surrounding sequence (28). With reference to the extension step, when starting from an O-6-mG primer basepair, most eukaryotic pols combine relative high discrimination and low efficiency. In agreement to the published data by Blanco et al, Y505M represent a similar pattern as DNA pol λ, having a relativity high discrimination and relatively lower extension efficiency for the mutagenic pair O-6-mG/dT than the correct base pair O-6-mG/dC, allowing us to conclude that the in-vitro generated Y505 would be a best candidate to guarantee an error free extension step during by pass of O-6-mG (Figure S2A).

Conclusion

In the present study we show the RP-A, an essential component of the cellular DNA replication and DNA repair machineries could not only modulate the intrinsic miscoding ability of DNA pol λ but can also completely suppress the incorporation of nucleotides opposite O-6-mG in a concentration dependent manner. The hydrophobic substitution mutant of the highly conserved residue Y505 in the active site of DNA pol is essential for its fidelity. The Y505M mutant of the pol λ is the first DNA pol described to date, which is able to preferentially incorporate dCTP opposite O-6-mG.

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Conflict of interest statement. None declared.

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Figure Legend

Figure 1. RP-A inhibits the bypass of O-6.mG lesion by DNA polymerase λ.

Experiments were performed as described in Materials and Methods. The template sequence is indicated on top of each panel (A) Single nucleotide incorporation opposite control template by DNA pol λ wt (lanes 2-5). Lane 1: control in the absence of dNTP and lane 6 in the presence of all four dNTPs. (B) As in panel A but opposite O-6mG template. (C) Incorporation opposite O-6.mG by 40nM of DNA pol λ wt of dCTP (lanes 1-4) and dTTP (lanes 6-9) in the absence of (lanes 1 and 6) or in the presence of (lanes 2-4, 7-19) of different amounts of RP-A. Lane 0 and 5 are controls in the absence of dNTPs. (D) Effect of increasing amounts of RP-A on the incorporation of dCTP and dTTP opposite O-6-mG by DNA pol λ wt. (E) As in panel C but in the presence of increasing amounts of PCNA.

Figure 2. DNA synthesis activity by DNA polymerase λ wt and Y505M.

(A) The sequence of human pol λ has been aligned to human pol β, μ and TdT. The tyrosine residue considered in this study is highlighted in the rectangular box and the respective substitution made by site-directed mutagenesis is indicated on the top. Conserved residues are indicated in bold. Multiple sequence alignment of the conserved Tyrosine residue in DNA pol λ from human, monkey, dog, cattle, rat, mouse, chicken, frog and zebra fish are shown on the left side. The conserved tyrosine residue is highlighted in the rectangular box and the conserved residues are indicated in bold. (B) The sequence of the template strand is indicated on top of each panel. Primer extension assays were performed as described in Materials and Methods section the presence of 5′ labeled 39/72-mer template. Titration of DNA pol λ wt (lanes 2-5), Y505M (lanes 7-10) in the presence of 10μM dNTPs. Lanes 1and 6: control reactions in the absence of nucleotides. (C) Variation in the initial velocities of the reaction catalyzed by 40nM DNA pol λ wt and Y505M on the undamaged template in the presence of dNTP as a function of the nucleotide substrate. Values are the mean of two independent experiments. Error bars are ± SD.

Figure 3. Translesion synthesis by DNA polymerase λ wt and Y505M.

The sequences are as indicated on the top of each panel. The experiments were carried out as indicated in Materials and Methods. (A) Single nucleotide incorporation opposite O-6mG damaged template by DNA pol λ wt (lanes 2-5) and Y505M (lanes 7-10) or pol β (lanes 12-15). Lane 1 is control reaction in the absence of dNTPs and lanes 6 and 11 in the presence of all dNTPs. (B) As in panel A, but in the presence of undamaged dG. (C) Variation in the initial velocities of the reaction catalyzed by 40nM DNA pol λ wt and Y505M as a function of the nucleotide substrate concentration. Values are the mean of two independent experiments. Error bars are ± SD (D) Incorporation of dTTP opposite dG by human DNA pol λ wt (lanes 2-4) and Y505M (lanes 6-8) at the indicated concentration. Lanes 1 and 5 are control reactions in the absence of dTTP. (E) Single nucleotide incorporation opposite the O-6mG lesion template by Y505A.

Figure 4.TLS by pol λ wt and Y505M over 8-oxo-G and 8-oxo-A.

Experiments were performed as described in materials and methods. The template sequence is as indicated on top of each panel. (A) Single nucleotide incorporation opposite 8-oxo-G by pol λ wt (lanes 2-5) and Y505M (lanes 7-10). Lanes 6 and 11 are in the presence of all four dNTP's. (B) Incorporation opposite 8-oxo-A. As in panel A. Lane 1 control reaction in the absence of dNTP's.

Figure 5. The fidelity of 6-O-mG bypass by DNA polymerase λ wt and Y505M is not influenced by the sequence context.

Experiments were performed as described in Materials and Methods. The template sequences are indicated on top of each panel. (A) Relative dCTP incorporation opposite the indicated template by DNA pol λ wt and Y505M at 0.1 μM and 1 μM. The product length is indicated on the bottom axis. (B) Relative dTTP incorporation opposite the indicated template by DNA pol λ wt and Y505M at 1μM and 10μM. The product length is indicated on the bottom axis. (C) Relative dTTP incorporation opposite the template indicated by pol λ wt and Y505M at 0.1 μM and 1 μM (D) Relative dTTP incorporation opposite the

indicated template by DNA pol λ wt and Y505M at 1μM and 10μM. All the values were obtained from 3 independent experiments. Error bars are ± SD.

Figure 6. RP-A inhibits the error free bypass of O-6-mG lesion by DNA polymerase λ. Experiments were performed as described in Materials and Methods. The template sequence is indicated on top of each panel. (A) Incorporation opposite O-6.mG by 40nM of DNA pol λ Y505M of dCTP (lanes 1-4) and dTTP (lanes 6-9) in the absence of (lanes 1 and 6) or in the presence of (lanes 2-4, 7-19) of different amounts of RP-A. Lane 0 and 5 are controls in the absence of dNTPs. (B) Effect of increasing amounts of RP-A on the incorporation of dCTP and dTTP opposite O-6-mG by DNA pol λ wt. (C) As in panel A but in the presence of increasing amounts of PCNA.

Table 1: Steady state kinetic parameters for nucleotide incorporation on the undamaged template by wt DNA polymerase λ and the Tyr505Met mutant[1]

DNA pol λ	dNTP		
	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1}min^{-1}$)
wt	**0.2122**	**0.023**	**0.108**
Tyr505Met	**0.1409**	**0.0061**	**0.043**

[1](K_{cat}/K_m) stands for the catalytic efficiency displayed by DNA pol λ wt and the mutant Tyr505Met for the insertion of dNTP.

Table 2: Steady state kinetic parameters for nucleotide incorporation on the O-6-mG template by DNA polymerase λ wt and the Tyr505Met mutant[1]

DNA pol λ	dCTP			dTTP		
	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1}min^{-1}$)	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1}min^{-1}$)
wt	**0.18**	**0.013**	**0.07**	**0.04**	**0.008**	**0.2**
Tyr505Met	**0.024**	**0.015**	**0.624**	**n.d.** [2]	**n.d.** [2]	**n.d.** [2]

[1](K_{cat}/K_m) stands for catalytic activity displayed by DNA pol λ wt and the mutant Tyr505Met for the insertion of the indicated nucleotide.
[2]n.d. non detectable incorporation of dTTP

Supplementary:

Figure S1A. The fidelity of 6-O-mG bypass by DNA polymerase λ wt and Y505M is not influenced by the sequence context.

Experiments were performed as described in Materials and methods. The template sequence are indicated on top of each panel. (A) Relative dCTP (lanes 2-4) and dTTP (lanes 6-8) incorporation opposite O-6-mG by DNA pol λ wt. dCTP (lanes 10-12) and dTTP (lanes14-16) incorporation opposite O-6-mG by Y505M. Lanes 1,5,9,13 are control reactions in the absence of dNTPs. (B) Relative dCTP (lanes 18-20) and dTTP (lanes 22-24) incorporation opposite O-6-mG by DNA pol λ wt. dCTP (lanes 26-28) and dTTP (lanes

30-32) incorporation opposite O-6-mG by Y505M. Lanes 17.21,25 and 29 are control reactions in the absence of dNTP′s.

Figure S2A. Extension of mismatched primers containing O-6-mG lesion by DNA polymerase λ wt and Y505M.

Experiments were performed as described in Materials and methods. The template sequences are indicated on top of each panel. (A) O-6-mG:C template – dCTP incorporation by DNA pol λ wt (lanes 6-8) and Y505M (lanes 2–4). Lanes 1 and 5 are control reactions in the absence of dCTP. (B) O-6-mG:T template – dCTP incorporation by DNA pol λ wt (lanes 14-16) and Y505M (lanes 10-12). Lanes 9 and 13 are control reactions in the absence of dCTP.

Figure 1

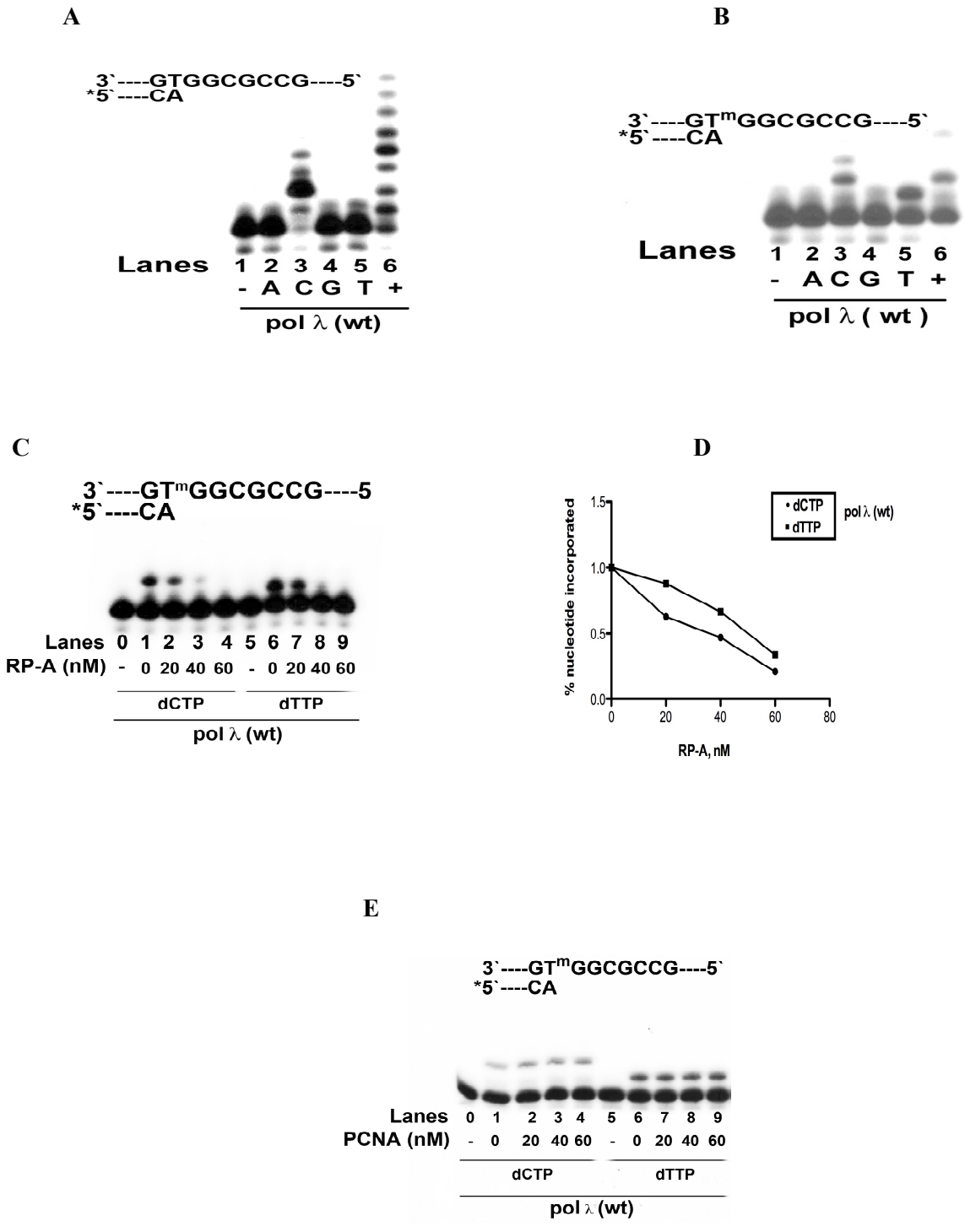
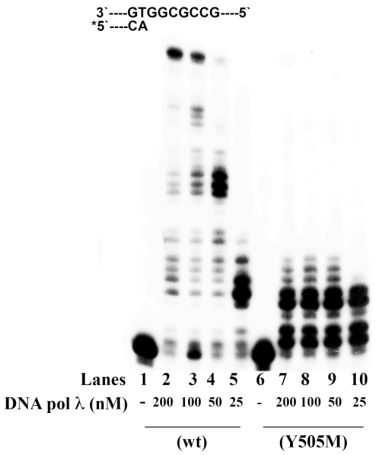

A
3`----GTGGCGCCG----5`
*5`----CA
Lanes 1 2 3 4 5 6
- A C G T +
pol λ (wt)
B
3`----GTmGGCGCCG----5`
*5`----CA
Lanes 1 2 3 4 5 6
- A C G T +
pol λ (wt)
C
3`----GTmGGCGCCG----5
*5`----CA
Lanes 0 1 2 3 4 5 6 7 8 9
RP-A (nM) - 0 20 40 60 - 0 20 40 60
dCTP dTTP
pol λ (wt)
D
% nucleotide incorporated
1.5
1.0
0.5
0.0
0 20 40 60 80
RP-A, nM
dCTP
dTTP
pol λ (wt)
E
3`----GTmGGCGCCG----5`
*5`----CA
Lanes 0 1 2 3 4 5 6 7 8 9
PCNA (nM) - 0 20 40 60 - 0 20 40 60
dCTP dTTP
pol λ (wt)

Figure 2

A

Human pol λ	501	C	A	L	L	Y	F	T	G	S 509
Monkey pol λ	501	C	A	L	L	Y	F	T	G	S 509
Dog pol λ	511	C	A	L	L	Y	F	T	G	S 519
Cattle pol λ	501	C	A	L	L	Y	F	T	G	S 509
Rat pol λ	499	C	A	L	L	Y	F	T	G	S 507
Mouse pol λ	499	C	A	L	L	Y	F	T	G	S 507
Chicken pol λ	501	C	A	L	L	Y	F	T	G	S 509
Frog pol λ	503	C	A	I	M	Y	F	T	G	S 511
Zebra fish pol λ	493	C	A	L	L	Y	F	T	G	S 501

B



C

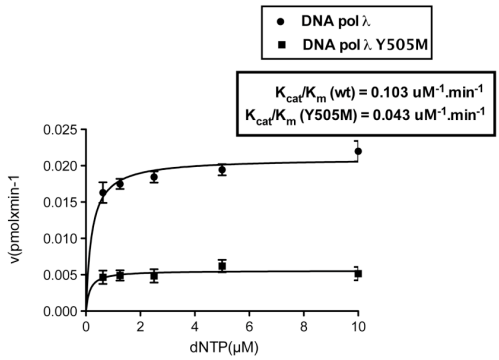
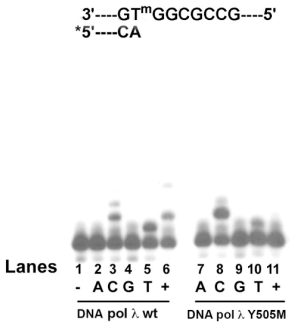
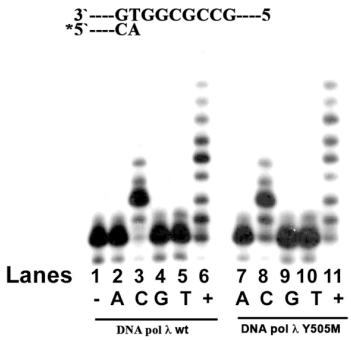


Figure 3

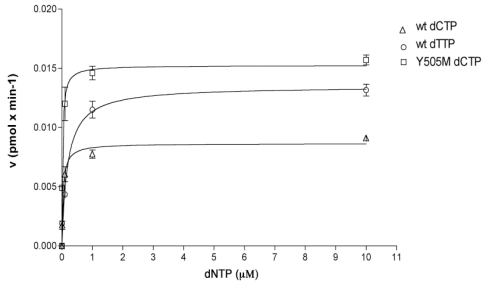
A



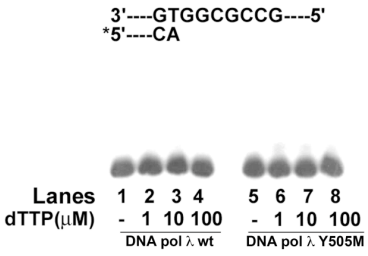
B



C



D



E

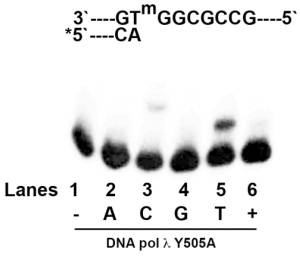
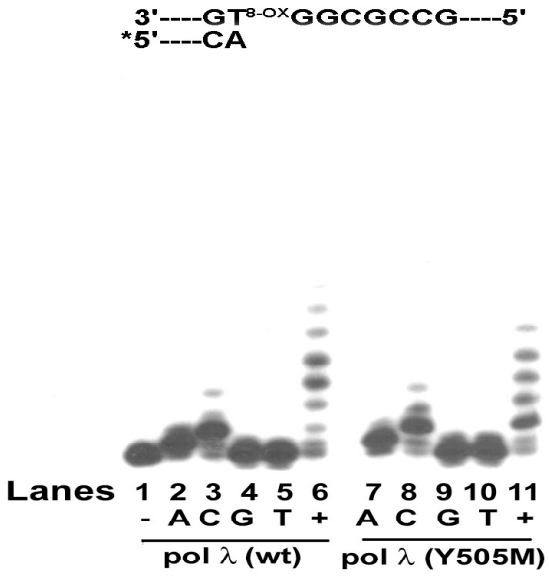


Figure 4

A


3'-----GT8-OXGGCGCCG-----5'
*5'-----CA
Lanes 1 2 3 4 5 6 7 8 9 10 11
- A C G T + A C G T +
pol λ (wt)
pol λ (Y505M)

B

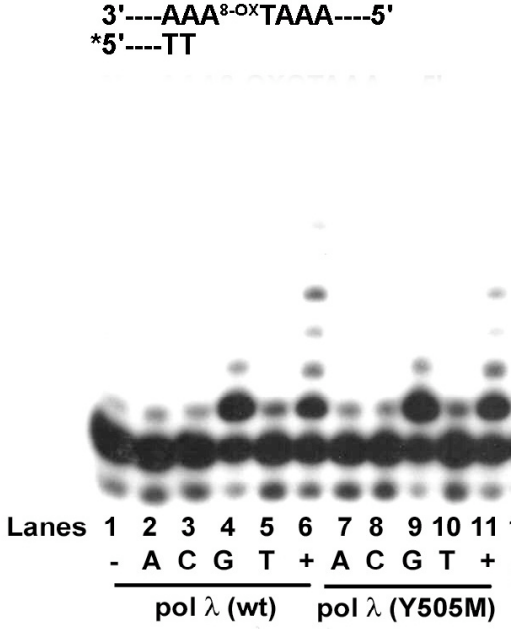

3'----AAA8-OXTAAA----5'
*5'----TT
Lanes 1 2 3 4 5 6 7 8 9 10 11
- A C G T + A C G T +
pol λ (wt)
pol λ (Y505M)

Figure 5

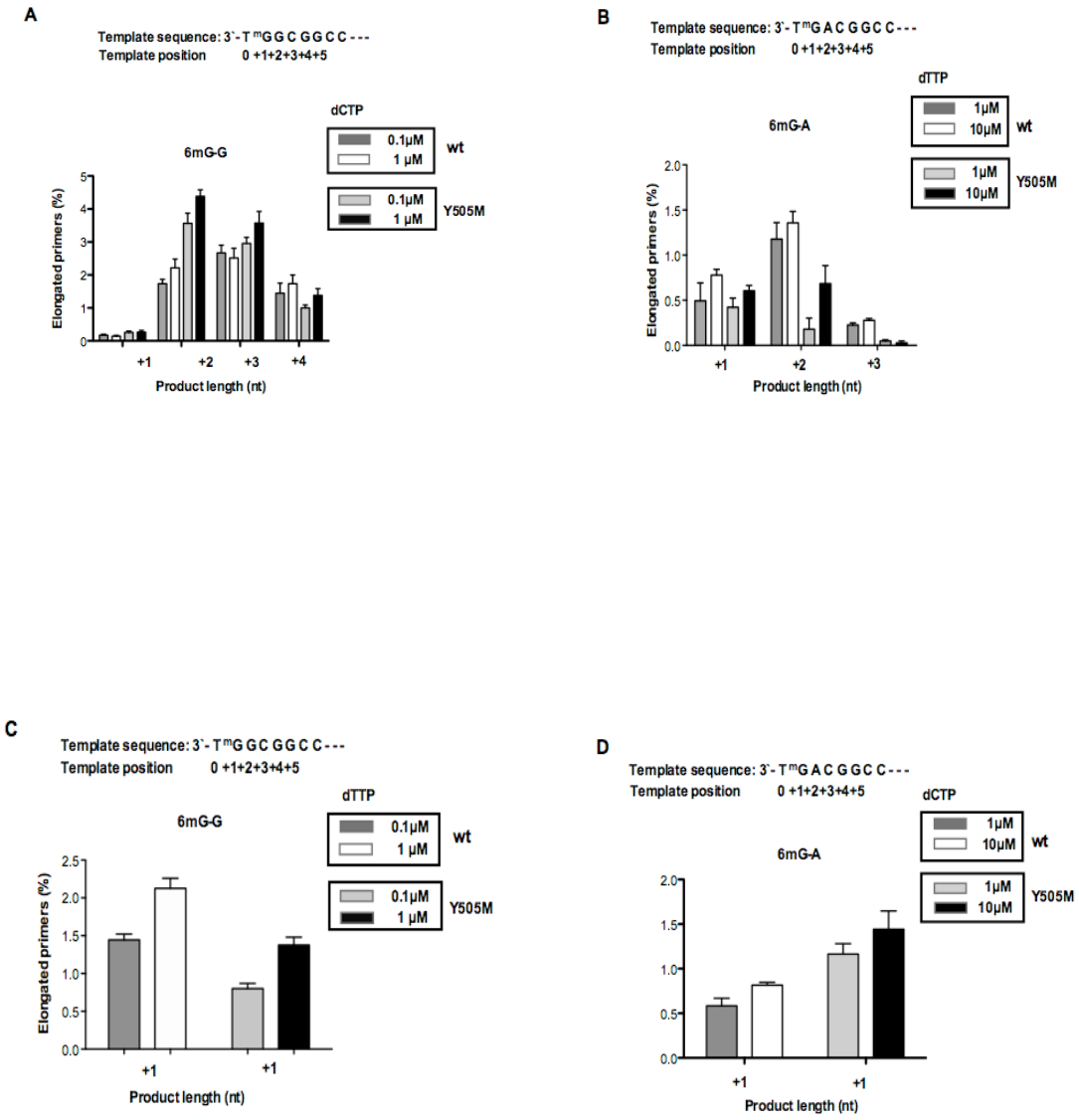
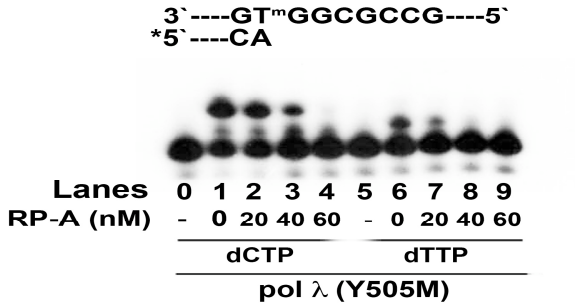
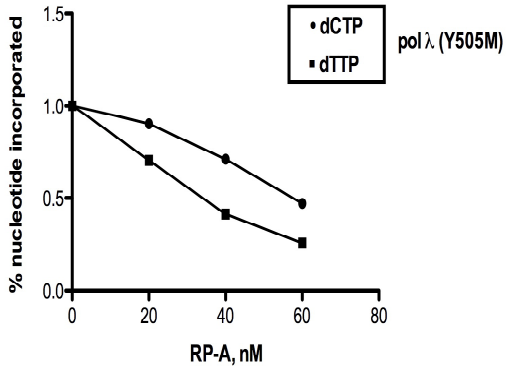

A
Template sequence: 3`- T mG G C G G C C - - -
Template position 0 +1+2+3+4+5
6mG-G
dCTP
0.1µM
1 µM
wt
0.1µM
1 µM
Y505M
Elongated primers (%)
Product length (nt)
B
Template sequence: 3`- T mG A C G G C C - - -
Template position 0 +1+2+3+4+5
6mG-A
dTTP
1µM
10µM
wt
1µM
10µM
Y505M
Elongated primers (%)
Product length (nt)
C
Template sequence: 3`- T mG G C G G C C - - -
Template position 0 +1+2+3+4+5
6mG-G
dTTP
0.1µM
1 µM
wt
0.1µM
1 µM
Y505M
Elongated primers (%)
Product length (nt)
D
Template sequence: 3`- T mG A C G G C C - - -
Template position 0 +1+2+3+4+5
6mG-A
dCTP
1µM
10µM
wt
1µM
10µM
Y505M
Product length (nt)

Figure 6

A


3`----GTmGGCGCCG----5`
*5`----CA
Lanes 0 1 2 3 4 5 6 7 8 9
RP-A (nM) - 0 20 40 60 - 0 20 40 60
dCTP
dTTP
pol λ (Y505M)

B


% nucleotide incorporated
1.5
1.0
0.5
0.0
0
20
40
60
80
RP-A, nM
dCTP
dTTP
pol λ (Y505M)

C

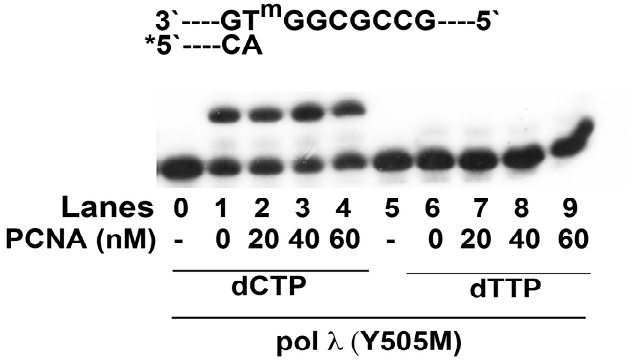

3`----GTmGGCGCCG----5`
*5`----CA
Lanes 0 1 2 3 4 5 6 7 8 9
PCNA (nM) - 0 20 40 60 - 0 20 40 60
dCTP
dTTP
pol λ (Y505M)

Figure S1A

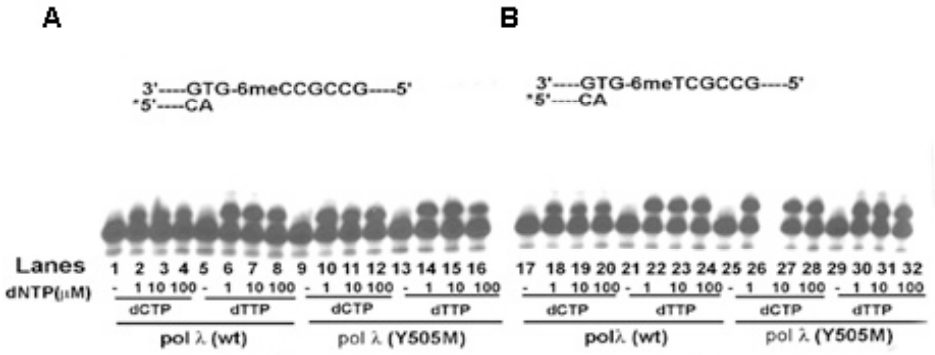
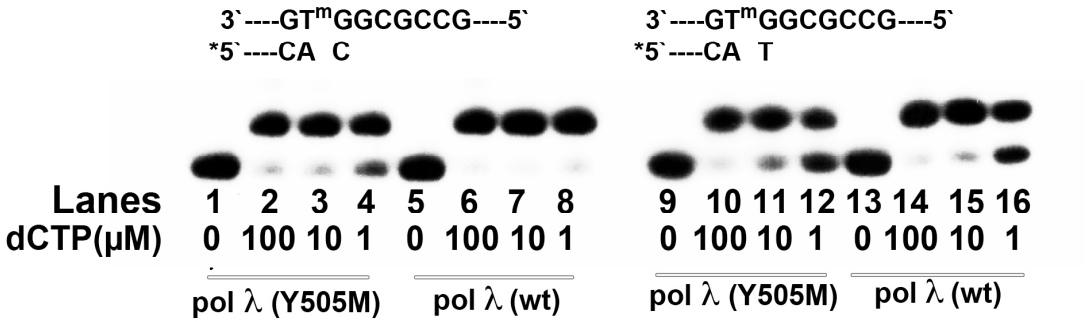


Figure S2A



Part II

AIM

Theories postulate that aging is triggered by cellular accumulation of oxidative DNA damage. Although more than 20 base lesions have been identified, 8-oxo-G is of special interest since it forms the high level of oxidative stress leading to mutations and eventually to cancer. DNA polymerase λ a member of the X family allows for the repair of this lesion via the BER pathway. The Werner syndrome protein (WRN), a member of the RecQ family of helicases, associates with BER proteins. The aim of the second part of the project was to understand a possible functional interplay of DNA polymerase λ and WRN in repair of oxidative damage.

INTRODUCTION

RecQ helicases

RecQ Helicases are one of the major proteins that are involved in maintaining genomic stability. RecQ proteins are highly conserved DNA helicases involved in DNA metabolic processes like DNA recombination, replication, and repair. Five different RecQ helicases have been identified in mammals, including humans. In contrast in *Escherichia coli*, *Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe*, only one RecQ helicase has been identified. Human RecQ helicase are named RECQ1, Bloom (BLM), Werner (WRN), RECQL4 and RECQL5 (133).

Genetic Disorder of RecQ helicase

Human RecQ helicases results in rare genetic disorders that lead to aging and cancer instability. The Werner syndrome (WS) and Bloom syndrome (BS) are associated with slow growth, abnormal facial features, infertility, and high onset of age-related diseases. WS patients are susceptible to early onset of sarcomas and mesenchymal tumors. BS patients have an early onset towards cancer. Individuals with mutation in the RecQ4 are prone to the Rothmund-Thomson syndrome (RTS). Pateins with RTS display growth deficiency, photosensitivity with poikilodermatous skin change, early graying and hair loss, jvenile cataracts and oesteogenic sarcomas. This leads to the RAPADILINO or the BALLER-Gerold syndrome. Genetic disorders associated with RecQ1 and RecQ5 mutations, have not been identified so far (134,135).

Properties of the WRN protein

The WRN gene encodes a protein of 1432 amino acids with a molecular weight of 163kDa.WRN contains a 3′→ 5′ exonuclease domain, an acidic region, a 3′→ 5′ helicase

domain, a RecQ C-terminal (RQC) domain, a helicase domain, a ribonuclease D C-terminal domain (HRDC) and finally a nuclear localization domain (136,137) (Figure 9).

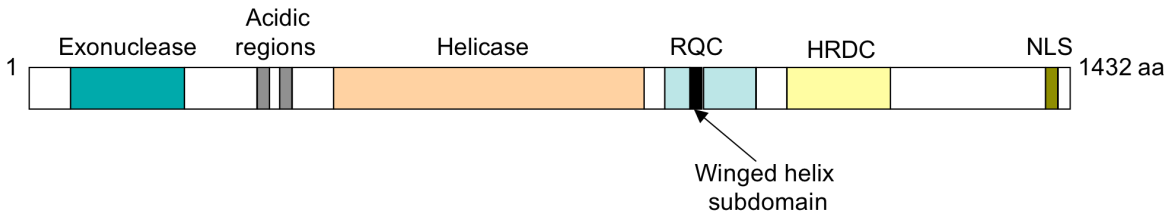


Figure 9. **Domain organization of the WRN protein.** A RQC, RecQ conserved C-terminal domain; HRDC, helicase and RNase D, C-terminal domain; NLS, nuclear localization signal. Reproduced from Bohr.V.A. Experimental Gerontology, **42**, 876-878 (2007).

WRN is a DNA structure specific helicase (138,139). The DNA G-quadruplex and triple helix are most preferred along with recombination intermediates like D-loops, holiday junction and three way junctions along with DNA replication structure like bubbles, forks and flaps (140-142). Single substitution mutation at K577M lacks ATPase activity and also the helicase activity. Unlike other RecQ helicase WRN processes a unique 3′→ 5′exonuclease activity in the N-terminal region, mutation at residue E84A inactivates the exonuclease activity of WRN (143). The exonuclease can preferentially digest dsDNA with a 5′ over-hang, and substrates with blunt-ended dsDNA such as replication fork like structures, holiday junctions, or D-loops (144,145). The WRN exonuclease activity is coordinated in a Zn^{2+} dependent manner (146). The human WRN protein is concentrated in the nucleolus,this is in contrast to the mouse WRN which is localized in the nucleoplasm (147). WRN depleted primary fibroblast show retarded S and/or G2/M progression (148). WRN cells are selectively sensitive to the DNA damaging agents 4-nitroquiniline 1-oxide, camptothecin, hydroxy urea and are mildly sensitive to γ irradiation induced DSBs. Therefore it plays a role in DNA repair (149,150). Oxidative DNA lesions are known to accumulate in the H_2O_2 treated diploid WS fibroblasts (151).

Post-translational modifications of the WRN protein

WRN is phosphorylated at serines, threonines, and tyrosines residues when cells are exposed to bleomycin and other replicative stress (152,153). Phosphorylation of Werner is dependent on the ataxia telangiectasia mutant protein (ATM) and the DNA PKc complex. C-Abl, a tyrosine protein kinase is also known to phosphorylate WRN (153). WRN is furthermore sumoylated *in vitro* and *in vivo*, though the functional consequence is yet to be understood (154). In addition to sumoylation, acetylation and tyrosine phosphorylation of WRN in nuclear trafficking (133). Acetylation of WRN by the transcriptional co-activator p300 is likely important in translocation of WRN from the nucleolus to nuclear foci (155,156).

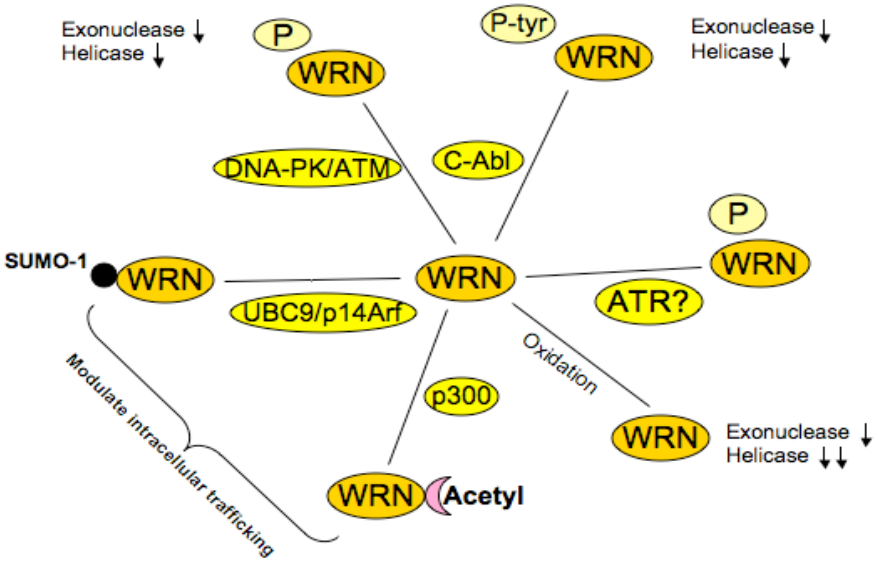


Figure 10. WRN posttranslational modifications. Post translational modification of WRN include: Phosphorylation on Ser/Thr or Tyr, sumoylation, acetylation and oxidation. The mediators and modifiers of WRN post-translational modifications are indicated. Functional effects of posttranslational modification are indicated. Two arrows indicate a stonger reaction than one arrow. Reproduced from Bohr.V.A, Trends in Biochem Sci, **33**, 609-620 (2008).

The BER pathway

Reactive oxygen species (ROS) are constantly generated in living organisms. The DNA bases are sensitive to oxidative DNA damage like the 8-oxo-G (157). The 8-oxo-G is a highly mutagenic miscoding lesion that can lead to G:C to T:A transversion mutations (158,159). There are two sub pathways of BER namely short patch (SP)-BER and long patch (LP)-BER. During SP-BER, only a few nucleotides are incorporated in the place of the damaged base; during LP-BER, two to six new nucleotides are incorporated. Specific DNA glycosylase, cleaves the N-glycosyl bond between the sugar and the base, releasing the damaged base to from an abasic site, termed apurinic/apyrimidinic site (160,161). There are several different glycosylase specific for certain lesions. DNA glysocylase are classified as mono- or bifunctional, depending on their reaction mechanisms. Monofunctional glycosylase (having only glysocylase activity) like UNG require another enzyme called the APE1 for the incision of the resulting abasic sugar residue leaving behind a 5′-deoxyribose phosphate (dRP). If the glycosylase is bifunctional having both glycosylase and AP-lyase activity such as OGG1 or NEIL1, then both base excision and an incision 3′ to the AP site occurs resulting in a one nucleotide gap that harbors a 3′-α, β-unsaturated aldehyde or 3′ phosphate. These one nucleotide gap requires the removal altered 3′-terminal groups prior to polymerization and/or ligation. The 3′-phosphoglycolate groups generated during direct SSBs can also be removed by APE-1 (162,163). After removal of the obstructive terminal or APE1 incision 5 to the AP site replacement of the excised nucleotide is performed by DNA pol β or DNA pol λ as a backup mechanism (164). This enzyme also removes the 5′dRP group left behind by the APE1 incision. If the 5 terminals are refractory to this DNA pol β AP lyase activity, strand displacement synthesis is required for incorporation of multiple nucleotides. During LP-BER several enzymes like PCNA, FEN1, DNA pol β and DNA pol δ/ε coordinate to fix the lesion. The final step involves the sealing of the nick by either DNA Ligase I (LP-BER) or DNA Ligase III/ XRCC1 (SP-BER) (165).

Role of the WRN protein in BER

BER is involved in repairing oxidative DNA lesions like the 8-oxoG and formamidopyrimidines (166). Although WRN does not interact with human OGG1 its association with the DNA glycosylase NEIL 1, specific for repair of 8-oxo-G, is the early damage-sensing step of BER (167). Interaction between human apurinic/pyrimidinic (APE1) and WRN inhibits the WRN helicase activity preventing promiscuous unwinding (168). BLM and WRN interact with DNA pol β, stimulating the strand displacement activity and this is independent of the helicase activity of WRN and BLM (155,169,170). WRN efficiently stimulates FEN-1 cleavage, on a flap DNA structure, an intermediate generated during BER and branch-migrating double-flap substrates generating during replication (171). WRN deficiency leads to hyperactivation and rapid premature accumulation of protein carbonyls. High levels of oxidative stress and oxidative DNA damage correlates with increased risk of sarcomas (172). Although WRN is not considered to be directly involved in BER, an important role for WRN in BER is supported by the finding WRN-deficient cells accumulate 8-oxo-G (151).

WRN interacting proteins for a variety of DNA transactions:

WRN helicase interacts with several protein in DSBR. RAD51, a key player in the strand invasion event during HR, interact with WRN (173). RAD52 inhibits and enhances WRN helicase activity in a DNA structure dependent manner, whereas WRN increases the efficiency of RAD52-mediated strand annealing (174). RAD54, another key protein, co-localizes with WRN in response to replicative stress (175). WRN also associates with the MRE11-RAD50-NBS1 complex via NBS1 and BRCA1 (176,177). BRCA1 stimulates the helicase activity of WRN. WRN is also known to participate in NHEJ since it interacts with Ku70-Ku80 heterodimer, a primary mediator of NHEJ and with DNA-dependent protein kinase (DNA PK) (152). WRN plays a role in MMR pathways, by removing mismatched nucleotide incorporation by a DNA pol during replication or DNA repair. WRN stimulates the extension activity of DNA pol δ and the family Y translesion DNA pols like η, ι and κ

over a lesion free and a lesion DNA template. It is also known that the WRN exonuclease fails to bypass certain DNA lesions like 8-oxo-G (145,178,179). WRN moreover interacts physically and functionally with several key proteins involved in replication, especially with RP-A, which stimulates the helicase activity (180,181). PCNA is also another key factor in replication interacting with WRN (180). Chromatin assembly factor-1 and WRN are both involved in the maintainance of genome stability (182). In response to DNA-damaging signals, both these proteins relocate to sites where DNA synthesis occurs. The WRN syndrome protein is required for recruitment of chromatin assembly factor 1 following DNA damage (182). Furthermore WRN deficiency is associated with defect in telomere maintenance. Studies show that WRN is associated with TRF1 and TRF2, both components of the shelterin complex (183). POT1 (protection of telomerase 1) is an ssDNA-binding protein that binds with high affinity to telomere repeats and strongly stimulates WRN helicase activity (183-185). The carboxyl-terminal part of WRN and the extreme carboxyl terminus of p53 can inturn be a region that plays an important role in regulating the functional state of p53. A cross talk between p53 and WRN helps preventing the accumulation of aberrations that give rise to premature senescence leading to cancer (186). WRN also interacts with exonuclease 1 (EXO-1) aviating the exonucleotytic and endonucleolytic incision function of EXO-1 (187).

7,8-dihydro-8-oxo-guanine and DNA polymerase λ

ROS (Reactive oxygen species) are generated by normal cellular metabolism and by exogenous agents. It is known that not all but most of the DNA lesions repaired by BER are products of ROS attack. There are up to 10^3 to 10^4 lesions generated per cell per day, the most common being, 8-oxo-G (188). 8-oxo-G is a highly mutagenic miscoding lesion that can lead to G:C to T:A transversion mutations. 8-oxo-G retains the ability to engage in correct Watson-crick base pairs with C, but oxidation of G (at C8) converts a hydrogen bond acceptor (N7) to a hydrogen bond donor, allowing a stable Hoogsteen base pair to form between 8-oxo-G and A, which is not possible in undamaged DNA (189) (Figure 11).

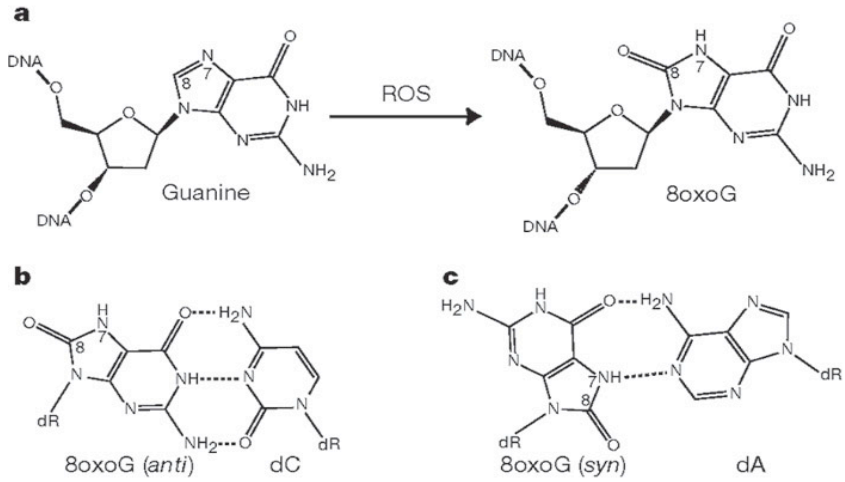


Figure 11. Models of base pairing for 8-oxo-G a.) Oxidation of guanine at C8 by reactive oxygen species (ROS). b.) 8-oxo-G in a Waton-Crick base pair with dC.dashed lines indicate potential hydrogen bonds. c.) 8-oxo-G (syn) in a Hoogsteen base pair with dA (anti). Reproduced from Hsu.G.W, Nature, **431**, 217-22(2004).

The BER pathway is the primary mechanism for the repair of oxidative base damage, such as 8-oxo-G and formamidopyrimidine. BER has alternative pathways depending on the damage and the responsive enzymes. The presence of 8-oxo-G on the replicative strand can lead to frequent misincorporation of A opposite 8-oxo-G by human replicative DNA pols like δ, ε and α at a higher frequency of 10-75%. Two different sub pathways of BER assure the complete repair of 8-oxo-G, when C:8-oxo-G mispair is detected on the transcribing strand, OOG1 dependent BER pathway is activated, leaving behind a lesion free intact DNA. This DNA strand than acts as a transcribing template. When A:8-oxo-G base pair is encountered; a MUTYH dependent pathway is stimulated, allowing for the removal of adenine. Finally, a key role of MUTYH and DNA pol λ in the repair of 8-oxo-G repair was recently shown by van Loon and Hübscher. DNA pol λ null mouse fibroblasts are known to be hypersensitive to oxidative DNA damaging agents, suggesting a vital role of DNA pol λ in protecting the cells against the cytotoxic effects of oxidative DNA damage (44). Auxiliary factors have known to play an important role in lesion bypass. PCNA and RP-A allowed correct incorporation of dCTP, opposite an 8-oxo-G template 1,200-fold more efficiently than the incorrect dATP by DNA pol λ and 68 fold by DNA pol η (43).

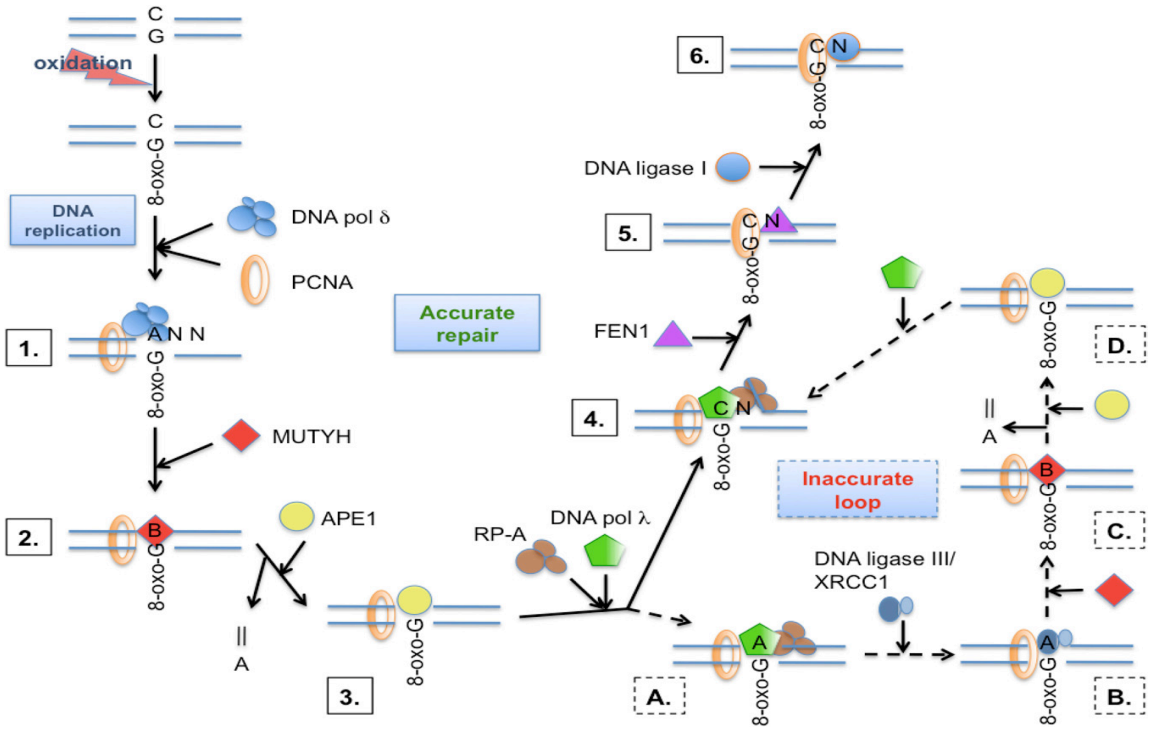


Figure 12. Model for MUTYH initiated long patch BER of 8-oxo-G after misincorporation by the replication machinery.1) DNA replication over an 8-oxo-G by DNA pol δ. 2.) recognition of an A:8-oxo-G mispair by MUTYH, removal of the A and formation of an AP site (denoted as B) 3.) Recruitment of APE1 mediated by MUTYH/PCNA and generation of 5`-P, 3`-OH gapped intermediate. 4.) Protection of 1-nt gap by RP-A and PCNA mediated recruitment of DNA pol λ, with accurate gap filling (dCTP incorporation) 5.) PCNA mediated recruitment of FEN 1 and removal of 1-nt flap. 6.) Ligation of the nick by recruited DNA Ligase 1 and futher faithfull OGG1 initiated DNA pol β mediated SP-BER of C:8-oxo-G product. Alternatively an inaccurate loop is initiated:(A) DNA pol λ catalyzes inaccurate gap filling.(B) recruitment of DNA Ligase III/XRCC1 mediated by PCNA and ligation of the nick. (C) Recognition of A:8-oxo-G mispair by MUTYH, remove of A and generation of APE1 mediated by MUTYH/PCNA, generation of 5-P, 3-OH gapped intermediate. This creates an opportunity for DNA pol λ to catalyze accurate LP-BER. Reproduced from van Loon, B and Hübscher, U, PNAS, **106**, 18201-18206 (2009).

PAPER II

Werner syndrome protein and DNA polymerase λ cooperate in the repair of oxidative DNA damage

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Running title: Interaction between WRN and DNA pol λ

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Abstract

Aging is associated with accumulation of DNA damage and increased incidence of cancer. Reactive oxygen species, constantly generated as by-products of cellular metabolism or from exogenous sources, readily attack genomic DNA generating mutagenic DNA lesions such as 7,8-dihydro-8-oxo-guanine (8-oxo-G), a major contributor to the ageing process. Here, we show that the Werner syndrome helicase (WRN), whose deficiency is associated with premature aging and cancer susceptibility, is specifically recruited to a DNA duplex containing an 8-oxo-G:A mispair in a manner dependent on DNA polymerase λ (DNA pol λ), which catalyzes accurate translesion synthesis over 8-oxo-G. Moreover, we demonstrate that WRN physically interacts with DNA pol λ and enhances its primer extension activity with a preference for DNA templates containing an 8-oxo-G. Experiments *in vivo* reveal that WRN and DNA pol λ colocalize at sites of 8-oxo-G lesions induced by H_2O_2. In addition, we have found that re-localization of WRN to 8-oxo-G lesions is dependent upon DNA pol λ. We propose that WRN facilitates the bypass of 8-oxo-G lesions by DNA pol λ during repair of 8-oxo-G:A mispairs, thus preventing GC to AT transversions which might lead to cancer and premature aging.

Introduction

Reactive oxygen species (ROS), that are constantly produced in living organisms as byproducts of normal cellular metabolism or as a consequence of environmental exposure to various physical and chemical agents, can generate a variety of oxidized DNA bases that are highly mutagenic and hence compromise genomic stability and promote carcinogenesis {Valko, 2004 #17; Klaunig, 2004 #18; Klaunig, #19}. One of the most frequent oxidative lesions is 7,8-dihydro-8-oxo-guanine (8-oxo-G) with a steady-state level of 4-10 lesions per 10^6 DNA bases in normal human tissue {Collins, 1999 #1; Dizdaroglu, 2002 #3}. Replication of genomic DNA containing 8-oxo-G lesions frequently leads to the formation of 8-oxo-G:A mispairs giving raise to a G:C to A:T transversion mutations {Avkin, 2002

#2}. Interestingly, these transversions are the most predominant somatic mutations found in lung, breast, ovarian, gastric and colorectal cancers, suggesting that a failure to eliminate 8-oxo-G lesions can initiate tumorigenesis and drive tumor progression {Greenman, 2007 #20}.

Oxidized base lesions are primarily eliminated by base excision repair (BER) {Hazra, 2007 #26}. In mammalian cells, the repair of 8-oxo-G:A mispairs is achieved *via* two BER events that occur sequentially on the two DNA strands (reviewed in {van Loon, #21}). The first event is initiated by excision of the mispaired A residue by the MutY glycosylase homologue (MUTYH) in a reaction coordinated by PCNA {Takao, 1999 #22; Hayashi, 2002 #23; van Loon, 2009 #7}. This is followed by cleavage of the apurinic site (AP) by the AP endonuclease 1 (APE1) resulting in a DNA intermediate containing a one-nucleotide gap with a 3'-OH moiety {Yang, 2001 #24; van Loon, 2009 #7}. PCNA and RPA then govern the bypass of the 8-oxo-G lesion by the DNA polymerase (pol) λ, which in the presence of these two auxiliary factors preferentially incorporates dCMP opposite the lesion {Maga, 2007 #6; Maga, 2008 #25; van Loon, 2009 #7}. Following lesion bypass, RPA dissociates and PCNA (by binding to FEN1) allows removal of the 5'-single-stranded DNA (ssDNA) flap resulting from the strand displacement synthesis by DNA pol λ {van Loon, 2009 #7}. Finally, DNA ligase I interacts with PCNA loaded on the nick arising from FEN1 cleavage and seals it, creating the substrate for a second BER event, which leads to the elimination of the 8-oxo-G lesion {van Loon, 2009 #7}. 8-oxo-G paired with C is predominantly excised by the OGG1 glycosylase in a short patch BER reaction in which DNA pol β fills the one nucleotide gap opposite the lesion and the DNA ligase III/XRCC1 complex finally seals the nick {Hazra, 2007 #26}. However, repair of 8-oxo-G:A mispairs in extracts from $Ogg1^{-/-}$ mouse embryonic fibroblasts (MEFs) has been found to proceed with a similar efficiency as in wild-type cell extract, suggesting that 8-oxo-G in this two-step BER pathway might be excised by enzymes other than OGG1 {Dantzer, 2003 #27}.

In the recent past, a number proteins acting in BER, such as NEIL1 {Das, 2007 #15}, APE1 {Ahn, 2004 #8}, DNA pol β {Harrigan, 2003 #9; Harrigan, 2006 #10}, FEN1 {Brosh, 2001 #11}, PCNA {Rodriguez-Lopez, 2003 #12} and RPA {Brosh, 1999 #33}

have been shown to interact physically and functionally with the WRN helicase/exonuclease, which is mutated in Werner syndrome (WS), an autosomal recessive disorder characterized by premature aging, cancer predisposition and genomic instability {Rossi, #16}. Interestingly, WRN-deficient cells accumulate 8-oxo-G lesions at a much higher rate than WRN-proficient cells, suggesting that WRN plays a role in the cellular response to oxidative stress {Das, 2007 #15}. In this work we have considered the possibility that WRN might act in the repair of 8-oxo-G:A mispairs. By using human cell extracts, we show that WRN is specifically recruited to a DNA duplex containing an 8-oxo-G:A mispair in a manner dependent on DNA pol λ. Moreover, WRN forms a complex with DNA pol λ through direct binding to its pol X core domain and stimulates the bypass of 8-oxo-G lesions by DNA pol λ. At the cellular level, WRN and DNA pol λ colocalize at sites of 8-oxo-G lesions in response to H_2O_2 treatment. Most importantly, WRN is recruited to sites of 8-oxo-G lesions only in the presence of DNA pol λ in the cell. These findings provide a strong evidence for a role of WRN in the repair of 8-oxo-G:A mispairs in mammalian cells.

Results

WRN is Specifically Recruited to DNA Containing an 8-oxo-G:A Mispair in a Manner Dependent on DNA Polymerase λ. To explore the role for WRN in the repair of 8-oxo-G:A mispairs, we employed a reversible cross-linking assay established previously to monitor the recruitment of BER proteins to damaged DNA {Parsons, 2004 #44}. A 3′-biotinylated hairpin loop oligoduplex (27 bp) containing a single 8-oxo-G:A mispair and the corresponding lesion-free substrate {van Loon, 2009 #7} were incubated with HeLa whole cell extract in the presence of a cross-linking agent and Mg^{2+}. At different time points, cross-linked DNA-protein complexes were isolated using streptavidin beads, and after reversing the cross-links, bound proteins were analyzed by Western blotting. We observed a rapid, damage-specific recruitment of WRN to the DNA substrate (Fig. 1A). A robust damage-specific recruitment of DNA pol λ was also detected as previously reported

{van Loon, 2009 #7}. These results provide a strong evidence for the involvement of WRN in the repair of 8-oxo-G:A mispairs.

To address the possibility that DNA pol λ was required for the recruitment of WRN to the 8-oxo-G:A-containing DNA substrate, whole cell extracts from pol $\lambda^{-/-}$ and pol $\lambda^{+/+}$ mouse embryonic fibroblasts (MEFs) were subjected to the crosslinking assay. We found that in the absence of DNA pol λ, the binding of WRN to the 8-oxo-G:A substrate was impaired, suggesting that DNA pol λ recruits WRN to the sites of 8-oxo-G:A repair (Fig. 1B).

WRN and DNA Polymerase λ Interact Physically *in Vivo* and *in Vitro*. To examine whether WRN and DNA pol λ interact physically, an extract of HEK293T cells expressing Myc-tagged DNA pol λ was subjected to immunoprecipitation with anti-WRN antibody. We found that DNA pol λ co-immunoprecipitated with WRN in this experiment (Fig. 2A, lanes 3-4). In contrast, neither DNA pol λ nor WRN were detected in the immunoprecipitate obtained with control IgG (Fig. 2A). Interestingly, the level of WRN-DNA pol λ complex detected in the extract from cells treated with H_2O_2 was slightly higher than that in the extract from non-treated cells (Fig. 2A, compare lanes 3 and 4), suggesting that the formation of this complex might be stimulated by oxidative DNA damage.

To examine whether WRN and DNA pol λ interact directly, we performed a co-immunoprecipitation experiment with a mixture of purified recombinant proteins. We found that WRN and DNA pol λ co-precipitated with anti-WRN antibody, but not with control IgG, indicating a direct interaction between these proteins (Fig. 2B). In order to quantify the affinity of this interaction, surface plasmon resonance measurements on a BIAcore 3000 were carried out. Increasing concentrations of DNA pol λ ranging from 0 nM to 500 nM, were passed through a biosensor chip coated with WRN (Fig. 2C). Evaluation of association and dissociation curves gave an apparent dissociation constant of 0.4 nM, indicating a strong binding.

Next, we used a GST pull-down assay to map the region of WRN binding to DNA pol λ. DNA pol λ bound well to GST-$WRN_{949-1432}$ and GST-$WRN_{500-946}$, but not to GST-WRN_{51-499} or to GST alone (Fig. 2E). These results indicated that DNA pol λ interacted with the helicase domain of WRN (amino acids 500-946) as well as with the C-terminal region of WRN, which contains winged-helix domain, a binding site of a number of other proteins shown to interact with WRN (reviewed in {Lee, 2005 #29}). In order to map the WRN-binding site on DNA pol λ, co-immunoprecipitation experiments were performed with different deletion variants of DNA pol λ (Fig. 2D). A stable interaction of WRN with DNA pol $λ_{244-575,}$ but not with DNA pol $λ_{1-132}$ and DNA pol $λ_{133-244}$ was observed, suggesting that WRN binds to the catalytic core domain of DNA pol λ (Fig. 2D).

WRN Enhances the Bypass of 8-oxo-G Lesions by DNA Polymerase λ. In order to gain insight into the role of WRN in the repair of 8-oxo-G:A mispairs, we next evaluated the effect of WRN on the extension by DNA pol λ of a 39-nt primer annealed to a 72-nt template containing an 8-oxo-G lesion at the +1 position. WRN exerted a robust stimulatory effect on primer extension by DNA pol λ in a reaction being more efficient on 8-oxo-G template than on a lesion-free template (Fig. 3A and B, lanes 2-6). In contrast, stimulation of DNA pol λ was not observed with other RecQ helicases such as BLM and RECQ5, suggesting that the observed stimulation was specific for WRN (Fig. 3A and B, lanes 2, 7 and 8). Single nucleotide incorporation experiment confirmed that WRN facilitated bypass of the 8-oxo-G lesion by DNA pol λ enhancing the incorporation of both dCMP and dAMP opposite the lesion (Fig. 3C, lanes 10-18). On the lesion-free template, DNA pol λ incorporated only dCMP in the presence of WRN as it did in its absence, indicating that WRN does not affect the inherent fidelity of DNA pol λ (Fig. 3C, lanes 1-9). To test the specificity of the stimulatory effect of WRN on the primer extension by DNA pol λ over 8-oxo-G, four other lesions were tested: an apurinic site, a 4-methylthymine, a 6-methylguanine and a cis-platinum adduct. WRN did not stimulate the bypass of these lesions by DNA pol λ suggesting that the observed effect over 8-oxo-G is specific (Supplementary Fig. S1).

To address whether the enzymatic activities of WRN are required for the observed stimulation of DNA pol λ, we used previously established mutants of WRN defective either in the exonuclease (E84A) or in the helicase (K577M) activity {Huang, 1998 #35; Gray, 1997 #34}. Both mutants stimulated primer extension by DNA pol λ to the same extent as the wild-type WRN on both 8-oxo-G and lesion-free templates (Fig. 3D), suggesting that the observed stimulatory effect is due to the physical interaction between DNA pol λ and WRN and is not dependent on WRN catalytical activities.

WRN and DNA Polymerase λ Colocalize at Sites of Oxidative DNA Damage. To evaluate the response of WRN and DNA pol λ to oxidative DNA damage *in vivo*, we used the indirect immunofluorescence technique to study the spatial distribution of these proteins in U2OS cells after H_2O_2 treatment. In agreement with previous reports, WRN was observed to localize to the nucleolus in the majority of non-treated cells, whereas DNA pol λ showed a dispersed nuclear staining (Fig. 4A, *top row*). Upon treatment with 500 μM H_2O_2 for two hours, WRN and DNA pol λ each formed >10 distinct foci per nucleus in 45% and 65% of cells, respectively. Approximately in 30% of cells, WRN foci showed complete co-localization with DNA pol λ foci. In the remaining cells, a partial co-localization between WRN and DNA pol λ was observed. It is known that H_2O_2 causes not only oxidative base damages, but also single-strand and double-strand DNA breaks {Benitez-Bribiesca, 1999 #28}. Therefore it is possible that the foci of WRN and DNA pol λ that did not colocalize represent sites of other types of DNA damage.

In order to support that WRN and DNA pol λ co-localize at sites of oxidative damage, untreated and H_2O_2-treated cells were co-immunostained either for visualization of WRN and 8-oxo-G or for visualization of DNA pol λ and 8-oxo-G. The results indicated that WRN and DNA pol λ co-localized with the sites 8-oxo-G damage in ~40% and ~50% of cells, respectively (Fig. 4B). Collectively, these results provide evidence that WRN and DNA pol λ operate in the repair of 8-oxo-G lesions in human cells.

DNA Polymerase λ is Required for WRN Recruitment to Sites of 8-oxo-G Lesions *in Vivo*. To further explore the function of WRN and DNA pol λ in the cellular response to oxidative DNA damage, WRN was down-regulated in U2OS cells by RNA interference and cells were subjected to immunofluorescence staining for DNA pol λ and 8-oxo-G prior to and after H_2O_2 treatment. We found that WRN depletion did not abolish the accumulation of DNA pol λ at sites of 8-oxo-G lesions (Fig. 5A). A small increase of DNA pol λ foci was observed in WRN-deficient cells relative to WRN-proficient cells even in absence of H_2O_2 (Fig. 5B). This might be due to the elevated level of spontaneous oxidative DNA damage previously observed in WRN deficient cells {Von Kobbe, 2004 #14}.

Finally, we compared by immunofluorescence the response of WRN to oxidative stress in DNA pol $\lambda^{+/+}$ and DNA pol $\lambda^{-/-}$ MEFs. The results demonstrated that genetic ablation of DNA pol λ impaired the recruitment of WRN to sites of 8-oxo-G lesions (Fig. 5B). Interestingly, in DNA pol $\lambda^{-/-}$ MEFs, WRN formed numerous foci in response to H_2O_2 treatment (data not shown). These most probably represent sites of DNA double-strand breaks resulting from processing of oxidative DNA damage. Collectively, our data indicate that DNA pol λ is required for the re-localization of WRN to sites of 8-oxo-G lesions.

Discussion

The replicative DNA polymerases α, δ and ε have strong tendency to incorporate an A opposite an 8-oxo-G leading to a Hoogsteen base pair, which can give rise to GC to AT transversion (reviewed in {Hubscher, 2010 #45}). Recent studies in our laboratory have advanced the understanding of the molecular mechanism underlying the repair of 8-oxo-G:A mispairs in mammalian cells {Maga, 2007 #6; Maga, 2008 #25; van Loon, 2009 #7}. Cross-linking experiments with human cell extracts and model DNA substrates have revealed that MUTYH, DNA pol λ, PCNA, RPA, FEN1 and DNA ligases I or III are involved in the excision of the mispaired A nucleotide and its replacement with a dCMP {van Loon, 2009 #7}, a BER reaction, which has to take place prior to the removal of the 8-

oxo-G lesion by the 8-oxo guanine DNA glycosylase (OGG1) (reviewed in {van Loon, 2010 #21}). Here we provide evidence that the WRN protein participates in this process by serving as an additional auxiliary factor that promotes the bypass of the 8-oxo-G lesion by DNA pol λ. We show that WRN physically interacts with DNA pol λ and accumulates at sites of 8-oxo-G lesions in a DNA pol λ-dependent manner, suggesting that DNA pol λ recruits WRN to the sites 8-oxo-G:A repair. Interestingly, previous studies have shown that WRN physically interacts with FEN1 and strongly stimulates FEN1-catalyzed cleavage of 5' flap substrates {Brosh, 2001 #11}. This activity is essential to generate a ligateable 3′-OH end following strand-displacement synthesis by DNA pol λ during the repair of 8-oxo-G:A mispairs {van Loon, 2009 #7}. Thus, it is possible that, in addition to DNA pol λ stimulation, WRN promotes the endonucleolytic cleavage by FEN1 in this BER pathway.

Our study has shown that the helicase and exonuclease activities of WRN are dispensable for DNA pol λ stimulation by WRN. Similarly, the functional interaction of WRN with FEN1 was independent of the WRN catalytic functions {Brosh, 2001 #11}. These findings suggested that the stimulatory effect of WRN on these enzymes stems from direct protein interaction. It is possible that physical interaction between WRN and DNA pol λ triggers a conformational change in the polymerase, which alters its catalytic properties. Interestingly, the domain of WRN that mediates the functional interaction between WRN and FEN1 resides within the C-terminal portion of WRN that also mediates interaction with DNA pol λ {Brosh, 2001 #11}. It will be interesting to examine whether this domain, referred to as winged-helix domain, plays a role in the stimulatory effect of WRN on DNA pol λ.

Like WRN, PCNA has also been found to interact with the catalytic domain of DNA pol λ and enhance its primer extension activity on lesion-free and 8-oxo-G-containing DNA templates. {Shimazaki, 2002 #42; Maga, 2002 #38; Maga, 2007 #6}. However, the mechanism of PCNA action seems to differ from that of WRN. PCNA was found to increase the processivity of DNA pol λ possibly by stabilizing its binding to the template/primer junction {Maga, 2002 #38}. In contrast, WRN appears to enhance the catalytic activity of DNA pol λ in general and its translesion DNA synthesis in particular.

WRN, however, stimulated the incorporation of both dCMP and dAMP opposite the 8-oxo-G lesion to a similar degree. In contrast, PCNA was found to increase the incorporation rates of dCMP opposite an 8-oxo-G, but not of dAMP incorporation opposite the lesion. The faithful incorporation of dCTP was enhanced about 100 times when the optimal concentrations of PCNA and RPA were used either in a standing start primer extension over 8-oxo-G {Maga, 2007 #6} or by repairing a one nucleotide gap containing an 8-oxo-G {Maga, 2008 #25}. A similar selectivity by DNA pol λ in the presence of PCNA and RPA was also found when the MUTYH DNA glycosylase and APE1 removed an incorrect A incorporated by DNA pol δ {van Loon, 2009 #7}. In a next step, it will be interesting to explore the role of WRN in the reconstituted 8-oxo-G:A repair system recently established in our laboratory{van Loon, 2009 #7}.

It was reported that WRN could also stimulate DNA synthesis by the human DNA pol δ {Kamath-Loeb, 2000 #39} and by the Y-family translesion (TLS) pols η, ι and κ, whereas it did not affect the synthesis by DNA pols α, β and ε {Kamath-Loeb, 2007 #40}. As in the case of DNA pol λ, the stimulation of Y-family DNA pols was found to be specific to WRN since no stimulation was observed with other RecQ family members such as BLM or *E. coli* RecQ {Kamath-Loeb, 2007 #40}. WRN could also promote lesion bypass by TLS pols in a manner dependent on type of lesion. For example, addition of WRN to limiting amounts of DNA pol η stimulated primer extension activity on templates containing a cyclobutane pyrimidine dimer (CPD) or an 8-oxo-G, whereas only limited lesion bypass of methyl adducts was observed with DNA pol η {Kamath-Loeb, 2007 #40}. Similarly, we found that WRN stimulated DNA pol λ to bypass 8-oxo-G, but not an apurinic site, 4-methylthymine, 6-methylguanine and cisplatin. Most importantly, the extension activity of DNA pol η on CPD- and 8-oxo-G-containing templates was comparable with that observed on lesion-free DNA template. In contrast, we found that WRN increased primer extension activity of DNA pol λ on 8-oxo-G template more efficiently than on lesion-free template, suggesting a specific interaction of WRN with DNA pol λ during the 8-oxo-G during lesion bypass.

WRN deficiency has been found to be associated with a defect in cell proliferation arrest in response to oxidative stress and with accumulation of 8-oxo-G and Fapy-G lesions in the genomic DNA {Von Kobbe, 2004 #14; Das, 2007 #15}. Inherited mutations in the WRN gene cause Werner syndrome that is characterized by premature aging and cancer predisposition, phenotypes arising from persistent oxidative stress to genomic DNA {Rossi, #16; Maynard, 2009 #5}. Our work adds significant insight into the basis of these phenotypes and further highlights the importance of WRN for oxidative damage repair in mammalian genomes.

Materials and Methods

Antibodies and Proteins. Affinity purified rabbit polyclonal antibodies against human DNA pol λ and WRN were described previously {van Loon, 2009 #7; Saydam, 2007 #31}. Antibody against mouse DNA pol λ was a gift from G.L. Dianov (University of Oxford). Mouse monoclonal anti-WRN antibodies were purchased from BD Biosciences (cat. # 611169; used for immunobloting) and Abcam (ab 66601, used for immunofluorescence staining). Mouse monoclonal antibody against 8-oxo-G was purchased from Millipore (MAB3560, clone 483.15). Rabbit polyclonal antibodies against Myc-tag and TFIIH were from Santa Cruz. Recombinant human DNA pol λ protein was expressed and purified as described {Ramadan, 2003 #36}. His-tagged recombinant human DNA pol λ fragments were purified on Ni-NTA agarose (Invitrogen) as recommended by the manufacturer. Recombinant human WRN protein and its mutants were produced and purified as previously described {Orren, 1999 #30}. These protein preparations did not contain any contaminating DNA polymerase activity (Supplementary Fig. S2). RECQ5 and BLM proteins were purified as previously described {Garcia, 2004 #46; Kanagaraj, 2006 #47}.

Cell Culture Experiments. All cell lines (HeLa, U2OS, HEK293T, MEFs) used in this study were maintained in DMEM (Gibco) supplemented with 10 % fetal calf serum (Gibco) and streptomycin/penicillin (100 U/ml). Where required, H_2O_2 (Sigma) was diluted using

DMEM and added to cell cultures to a final concentration of 500 μM. Transfection of 293T cells with pcDNA3 vector expressing Myc-tagged human DNA pol λ {Wimmer, 2008 #32} was done using Metafectene (Biontex) according to manufacturer's instructions. Transfection of siRNA oligonucleotides was carried out using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Cells were analyzed 72 hours after siRNA transfection.

Immunoprecipitation Assays. Total cell extract preparation and immunoprecipitation (IP) were carried out as described previously {Saydam, 2007 #31}. Briefly, cell extracts (1.5 mg of protein) pre-treated with DNaseI (Roche) were incubated overnight at 4°C either with purified rabbit polyclonal anti-WRN IgGs (2 μg) or with IgGs purified from preimmune serum (2 μg). Mixtures were then incubated for 1.5 hours at 4°C with Protein A/G-agarose beads (20 μl; Santa Cruz). After extensive washing, the immune complexes were eluted from beads by heating at 95°C for 10 minutes in 2x Laemmli SDS buffer. The obtained immunoprecipitates were subjected to Western blot analysis. The blots were probed with appropriate antibodies and immune complexes were detected using the ECL-plus reagent (GE Healthcare). For IP of purified recombinant proteins, a mixture of equal amounts of WRN (500 ng) and DNA pol λ (500 ng) was incubated for 2 hours at 4°C and then added to Protein A/G-agarose beads (20 μl) coated with rabbit polyclonal anti-WRN IgGs (2 μg). After incubation for 2 hours at 4°C, the beads were washed and subjected to Western blot analysis.

Immunofluorescence Assays. Cells cultured on coverslides were fixed with 3.7% formaldehyde for 10 minutes at room temperature (RT) and subsequently permeabilized by soaking in 0.2% (v/v) Triton X-100 for 5 minutes at RT. After blocking in PBS containing 5 mg/ml BSA for 30 minutes at RT, the fixed cells were incubated overnight at 4°C with appropriate primary antibodies. All antibodies were diluted in blocking solution: rabbit polyclonal anti-WRN antibody (1:500), rabbit polyclonal anti-DNA pol λ (1:500) and mouse monoclonal anti-WRN antibody (Abcam, 1:50). The slides were washed with PBS

and incubated for 1.5 hour at RT with secondary antibodies diluted in blocking solution: FITC-conjugated sheep anti-rabbit IgG (Sigma; 1:700) and Texas Red-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, 1:200). After washing with PBS, coverslips were mounted on Vectashield (Vector Laboratories) and images were captured by an Olympus IX81 fluorescence microscope. At least 100 nuclei were analyzed in each experiment.

For simultaneous detection of WRN and 8-oxo-G, cells were fixed and sequentially incubated with rabbit polyclonal anti-WRN antibody and anti-rabbit FITC conjugated secondary antibody. Stained cells were then fixed with 100% cold methanol for 30 minutes at -20°C and immersed in 100% cold acetone for 30 seconds. After washing, the fixed cells were treated with 2M HCl for 30 minutes to denature the DNA and then neutralized with 0.1 M borate buffer (pH 8.5). After washing and blocking, cells were stained with mouse monoclonal anti-8-oxo-G (IgM) antibody (1:100) followed by Texas Red-conjugated donkey anti-mouse IgM secondary antibody (Jackson Immunoresearch, 1:150). After washing, coverslips were mounted and analyzed as described above. The same procedure was used for simultaneous detection of DNA pol λ and 8-oxo-G lesion.

GST Pull-Down Assays. GST pull-down assays were performed as previously described {Saydam, 2007 #31}. Briefly, bacterially expressed GST-WRN fusion proteins were bound to glutathione-Sepharose beads (20 μl; GE Healthcare) and incubated with 0.5 μg of recombinant human DNA pol λ for 2 hours at 4°C. After extensive washing, bound proteins were analyzed by Western blotting. Glutathione beads coated with GST-protein only were used as control.

Surface Plasmon Resonance measurements. The kinetics of binding of the human DNA pol λ to WRN was measured at 25°C using a BIAcore 3000 instrument (GE Healthcare). A total of 1000 resonance units (RU) of WRN were immobilized on a research grade CM5 chip (Bio core ^{TM}). Different concentrations of purified human DNA pol λ (0-500 nM)

were injected at 20 μl/min across the sensor surface. Data analysis was performed with the BIAevalution software (Pharmacia, version) (see *SI Text)*.

Cross-linking Assay. DNA-protein cross-linking assays with cell extracts and hairpin oligonucleotide substrates attached to streptavidin magnetic beads (Invitrogen) were performed as described previously {van Loon, 2009 #7}. Reactions were carried out in the presence of Mg^{2+} over different periods of time ranging from 0.25 to 4 minutes. Each reaction contained 100 μg of protein.

Primer Extension Assay. Annealing of 72mer containing 8-oxo-G lesion (or a normal G) with the 5'-[^{32}P]-labeled 39mer primer created a primer/template substrate with the lesion (or a normal G) at the +1 position relative to single-strand/double-strand junction (see SI text for sequences of the oligonucleotides). The reaction mixtures (10 μl) contained 50 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.25 mg/ml BSA, 10 μM dNTP, 1 mM $MgCl_2$ and 10 fmol of the 5'[^{32}P]-labeled primer/template substrate. Concentrations of human DNA pol λ and human WRN are indicated in figures and figure legends. Reaction mixtures were separated on a denaturing urea-polyacrylamide gel and radiolabeled DNA species were visualized by autoradiography.

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Figure Legends

Fig. 1. Recruitment of WRN to a DNA duplex containing an 8-oxo-G:A mispair is dependent DNA pol λ. (*A*) DNA-protein cross-linking assay using 8-oxo-G:A (*Left panel*) or G:C (*Right panel*) hairpin DNA substrates and HeLa whole-cell extract. (*B*) DNA-protein cross-linking assay using 8-oxo-G:A hairpin substrate and whole cell extracts from DNA pol $\lambda^{-/-}$ (*Left panel*) or DNA pol $\lambda^{+/+}$ (*Right panel*) mouse embryonic fibroblasts. The experiments were performed under the conditions specified in Materials and Methods. Blots were probed with mouse monoclonal anti-WRN and rabbit polyclonal anti-DNA pol λ antibody.

Fig. 2. WRN and DNA pol λ physically interact. (*A*) Co-immunoprecipitation of DNA pol λ with WRN from extracts of non-treated and H_2O_2-treated HEK293T cells. Cells were transiently transfected with plasmid expressing Myc-tagged DNA pol λ and, 48 hours post transfection, treated with 500 μM H_2O_2 for 2 hours or mock-treated. WRN was immunoprecipitated using rabbit polyclonal anti-WRN antibody as described under Materials and Methods. Blots were probed with rabbit polyclonal anti-Myc antibody and mouse monoclonal anti-WRN antibody. Lane 2, control immunoprecipitatate obtained with preimmune rabbit IgGs. (*B*) Co-immunoprecipitation of DNA pol λ with WRN from a mixture of purified proteins (300 ng of each protein) pre-incubated on ice for 2 hours. Blots were probed as in (*A*). (*C*) Analysis of WRN binding to DNA pol λ using BIAcore 3000. An overlay plot of sensograms obtained by injection of indicated concentrations of DNA pol λ over a CM-5 chip coated with WRN is shown. (*D*) Mapping of WRN-interacting domain of DNA pol λ. (*Top panel*) Domain organization of DNA pol λ. NLS, nuclear localization sequence; BRCT, BRCA1 C-terminal domain; Ser/Pro, serine/proline rich domain; Pol X domain, catalytic domain conserved in the X family of DNA pols. Black lines indicate fragments used for mapping. (*Bottom panel*) Co-immunoprecipitattion assay. Purified $(His)_6$-tagged DNA pol λ fragments were incubated with full-length WRN followed by immunoprecipitation with rabbit polyclonal antibody against WRN or with control (Ctrl) IgG. Immunoprecipitates were analyzed by Western blotting as in (*A*). IB,

immunoblot. (*E*) Mapping of the DNA pol λ-interacting domain of WRN. (*Top panel*) Domain organization of WRN. Exo, Exonuclease domain; DExH, helicase domain conserved in the DExH family of helicases; RQC, RecQ C-terminal domain composed of Zn-binding (Zn) and winged-helix (WH) subdomains; HRDC, helicase and RNase D C-terminal domain. The black lines indicate WRN fragments used. (*Bottom panel*) GST pull-down assay. The indicated WRN fragments were produced as fusions with GST and immobilized on glutathione beads. Binding of purified recombinant DNA pol λ to the beads was analyzed by Western blotting. The Coomassie Brilliant Blue-stained gel from SDS-PAGE analysis of the GST-WRN fragments isolated on glutathione beads is also shown. Asterisks indicate the GST-WRN fragments. The additional protein bands correspond to proteolytic degradation products as determined by immunoblotting using an anti-GST antibody. Lane 1, 20% of input of DNA pol λ.

Fig. 3. WRN stimulates 8-oxo-G bypass by DNA pol λ. (*A*) Extension by DNA pol λ of a 5′-end-labeled 39-nt DNA primer annealed to a 72-nt undamaged control DNA template in the presence of indicated concentrations of WRN, BLM and RECQ5, respectively. Individual dNTPs were present at a concentration of 10μM. Lane 1, control reaction in the absence of dNTPs. (*B*) As in (*A*) except that the DNA template contains a single 8-oxo-G at position +1. (*C*) Single nucleotide incorporation by DNA pol λ on the control and 8-oxo-G templates as shown in A and B. Reactions were carried out in the absence and in the presence of 10 nM WRN as indicated. Lane 1 control reaction in the absence of dNTPs. (*D*) Primer extension by DNA pol λ in the presence of helicase-deficient (K-WRN) and exonuclease-deficient (N-WRN) mutants of WRN. The DNA substrates used were the same as in (*A*) and (*B*), respectively. Reactions in (*A*-*D*) were carried out and analyzed as specified under Materials and Methods.

Fig. 4. WRN and DNA pol λ co-localize at sites of 8-oxo-G lesions in human cells. (*A*) WRN colocalizes with DNA pol λ after exposure of U2OS cells to oxidative stress. Cells grown on cover slides were either left untreated or treated with 500 μM H_2O_2 for 2 hours.

After treatment, cells were fixed and immunostained using anti-DNA pol λ (green) and anti-WRN (red) antibodies. DAPI staining of the nucleus is shown in blue. (*B*) WRN and DNA pol λ are recruited to sites of 8-oxo-G lesions. U2OS cells were left either non-treated (*Top panels*) or treated with 500 μM H_2O_2 for 2 hours (*Lower panels*), fixed and co-stained either with anti-WRN (green) and anti-8-oxo-G (red) antibodies or with anti-DNA pol λ (green) and 8-oxo-G (red) antibodies under conditions described in Materials and Methods. Images were captured on an Olympus IX81 fluorescence microscope

Fig. 5. WRN localization to sites of 8-oxo-G lesions *in vivo* is dependent on DNA pol λ. (*A*) Western blot analysis of extracts of U2OS cells transfected with WRN siRNA (siWRN) and control siRNA (siCtrl), respectively. Cells were harvested 72 hours post-transfection. Blots were probed with antibodies against WRN, DNA pol λ and TFIIH (loading control). (*B*) Graph showing the proportion of siCtrl and siWRN cells positive for colocalization between DNA pol λ and 8-oxo-G foci after mock- and H_2O_2-treatment. Cells were treated with 500 μM H_2O_2 (or mock-treated) for 2 hours, fixed and immunostained to visualize WRN and 8-oxo-G. Treatment was carried out 72 hours after siRNA transfection. (*C*) Western blot analysis of extracts from DNA pol $\lambda^{+/+}$ and DNA pol $\lambda^{-/-}$ mouse embryonic fibroblasts (MEFs). Blots were probed with antibodies against WRN, DNA pol λ and β-tubulin (loading control). The arrowhead indicates the band corresponding to mouse DNA pol λ. (*D*) Graph showing the proportion of DNA pol $\lambda^{+/+}$ and DNA pol $\lambda^{-/-}$ MEFs positive for co-localization of WRN and 8-oxo-G foci after mock- and H_2O_2-treatment, respectively. H_2O_2 was present at a concentration of 100 μM for 2 hours. After treatment, cells were fixed and stained for WRN and 8-oxo-G. The data points in (*A*) and (*B*) represent the mean of three independent experiments with at least 100 nuclei counted in each experiment. IB, immunoblot.

Supplementary Information

Supplementary Materials and Methods

DNA Substrates and siRNA. Oligonucleotides used for primer extension assays were purchased from Purimix and purified by denaturing PAGE. The sequences are:
72-mer template:
3′-ATGTTGGTTCTCGTATGCTGCCGGTCACGGCTTAAGTGT**X**GCGGCCGCG GTTGGAGGGCTTATAGATTATG-5′; the bold letter X denotes G, 8-oxo-G, abasic site, 4-methylthymine or 6-methylguanine.
60-mer template:
3′-ATGTTGGTTCTCGTATGCTGCCGGTCACGGCTTTTCTT**GG**TTCCTATCGG TGGTTAGTCG-5′; the 60-mer template containing a single cis-platinum adduct on the G residues indicated in bold was prepared as described {Hoffmann, 1995 #37}.
The underlined sequences correspond to the primer annealing sites. The primers were 5′-end labeled with T4 polynucleotide kinase and γ[^{32}P]ATP (GE Healthcare) mixed with appropriate template oligonucleotide at 1:1 (M/M) ratio in the presence of 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl, heated at 95°C for 10 minutes and then slowly cooled down to room temperature.

All siRNA oligoduplexes used in this study were purchased from Microsynth. The sequences of the top strands of these duplexes are shown below:
siWRN: 5'-UAGAGGGAAACUUGGCAAA-3'
siCtrl: 5'-CGU ACG CGG AAU ACU UCG A-3'

Surface Plasmon Resonance Measurements. All binding experiments were performed on a Biacore 3000 apparatus (Biacore, GE Healthcare) at 25°C in running HBS buffer (10 mM Hepes (pH 7.5), 150 mM NaCl, 3 mM EDTA and 0.005% P20 surfactant). A total of 1000 resonance units (RU) of WRN helicase (ligand) were immobilized on a research grade CM5 biosensor chip (BIAcore^{TM}) in 10 mM sodium acetate (pH 3.0) according to the

manufactures amine coupling kit. The un-reacted residues on the surface were blocked by washing with 1M ethanolamine (pH 8.5). One flow cell left blank was used as reference cell. Different concentration of DNA pol λ were prepared in HBS running buffer and injected at 20 μl/min across the sensor surface. The surface was regenerated by a 30 sec pulse of 10 mM glycine followed by a 30 sec pulse of 100 mM HCl and 0.01% SDS at a flow rate of 35 μl/min. The signal changes on the activated/blocked control channel were subtracted from the WRN-DNA pol λ binding interaction and the subtracted sensogram were analyzed. Injections were performed for each protein concentration. Sensograms were evaluated kinetically using BIAcore Evaluation Software.

Supplementary References

Supplementary Figure Legends.

Fig. S1. WRN has no effect on primer extension by DNA pol λ on DNA templates containing apurinic site, 4-methylthymine, 6-methylguanine or cis-platinum**.** The experiments were performed as described in Materials and Methods. DNA pol λ-catalyzed extension of a 5'-[^{32}P]-labeled 39-nt DNA primer annealed to 72-nt templates containing base lesions as indicated. Reactions were carried out in the absence or in the presence of indicated concentrations WRN. Lanes 1, 6, 11 and 16, control reactions carried out in the absence of DNA pol λ and WRN (no enzyme control). AP, apurinic site.

Fig. S2. Preparations of WRN and its mutants used in this study do not contain a contaminating DNA polymerase activity. Primer extension assay was carried out as described in Materials and Methods using the lesion-free template. WRN and its mutants were present at a concentration of 8 nM. DNA pol λ was 6 nM.

Figure 1

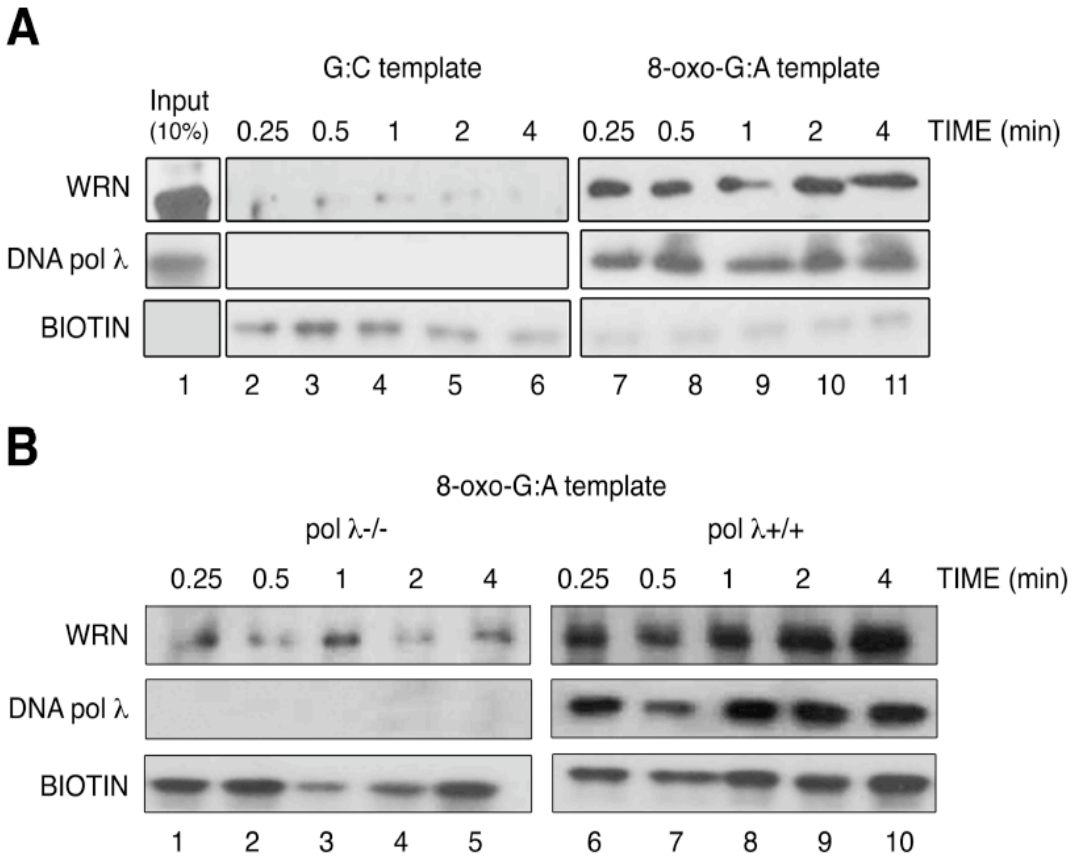

A
G:C template
8-oxo-G:A template
Input
(10%)
0.25 0.5 1 2 4
0.25 0.5 1 2 4
TIME (min)
WRN
DNA pol λ
BIOTIN
1 2 3 4 5 6 7 8 9 10 11
B
8-oxo-G:A template
pol λ-/-
pol λ+/+
0.25 0.5 1 2 4
0.25 0.5 1 2 4
TIME (min)
WRN
DNA pol λ
BIOTIN
1 2 3 4 5 6 7 8 9 10

Figure 2

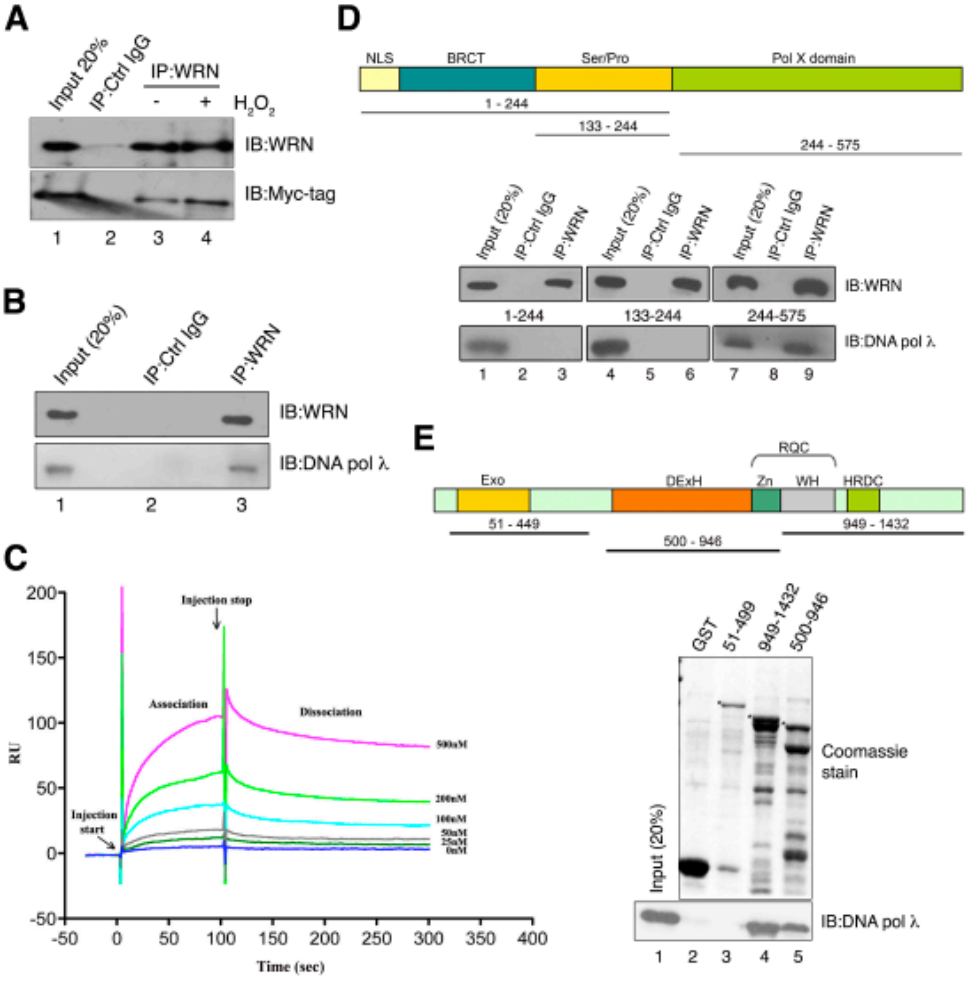

A
Input 20%
IP:Ctrl IgG
IP:WRN
- + H_2O_2
IB:WRN
IB:Myc-tag
1 2 3 4
B
Input (20%)
IP:Ctrl IgG
IP:WRN
IB:WRN
IB:DNA pol λ
1 2 3
C
Injection stop
Association
Dissociation
Injection start
RU
Time (sec)
500nM
200nM
100nM
50nM
25nM
0nM
D
NLS
BRCT
Ser/Pro
Pol X domain
1 - 244
133 - 244
244 - 575
Input (20%)
IP:Ctrl IgG
IP:WRN
IB:WRN
1-244
133-244
244-575
IB:DNA pol λ
1 2 3 4 5 6 7 8 9
E
RQC
Exo
DExH
Zn
WH
HRDC
51 - 449
500 - 946
949 - 1432
GST
51-499
949-1432
500-946
Coomassie stain
Input (20%)
IB:DNA pol λ
1 2 3 4 5

Figure 3

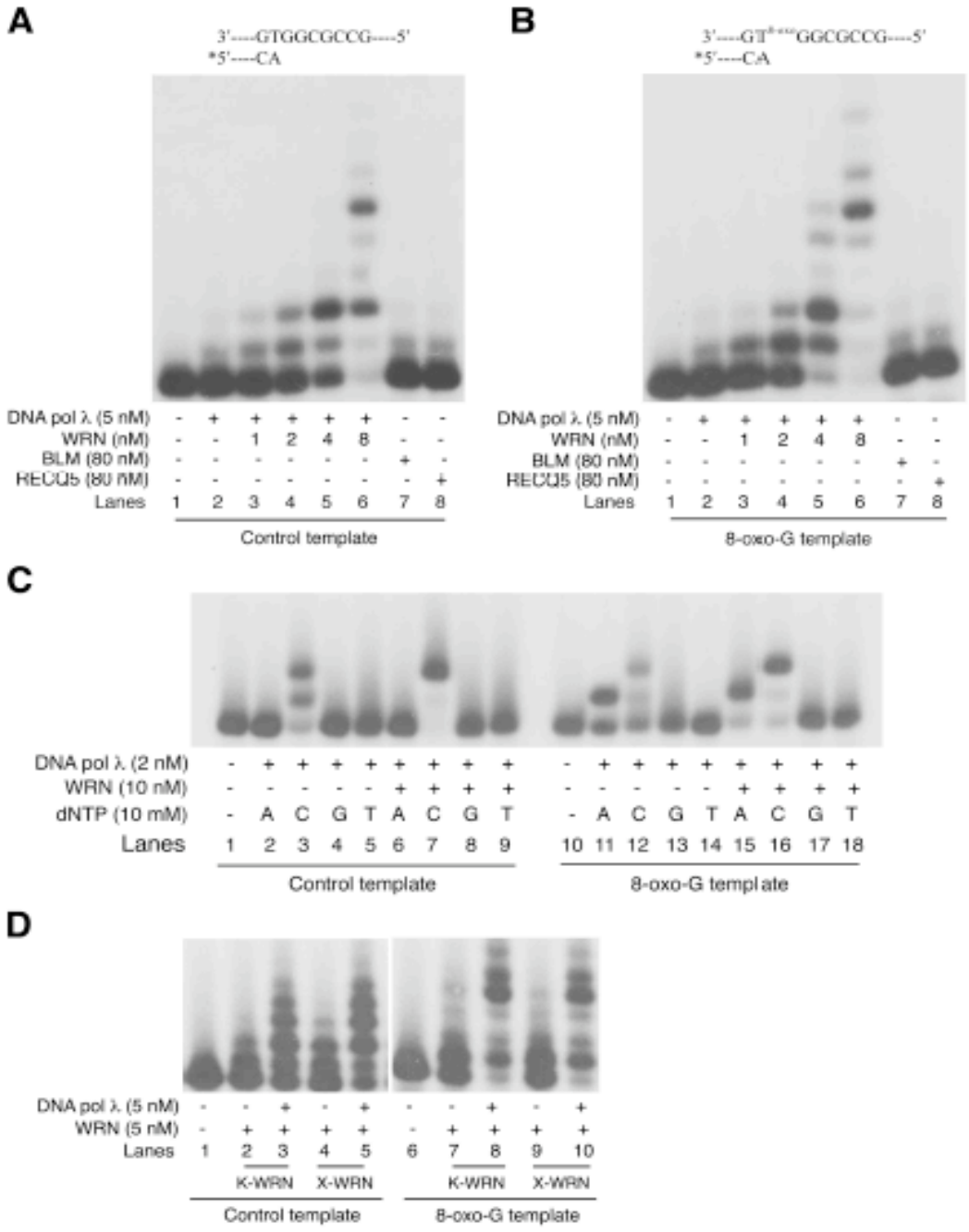

A
3'----GTGGCGCCG----5'
*5'----CA
DNA pol λ (5 nM) - + + + + + - -
WRN (nM) - - 1 2 4 8 - -
BLM (80 nM) - - - - - - + -
RECQ5 (80 nM) - - - - - - - +
Lanes 1 2 3 4 5 6 7 8
Control template
B
3'----GT*oxoGGCGCCG----5'
*5'----CA
DNA pol λ (5 nM) - + + + + + - -
WRN (nM) - - 1 2 4 8 - -
BLM (80 nM) - - - - - - + -
RECQ5 (80 nM) - - - - - - - +
Lanes 1 2 3 4 5 6 7 8
8-oxo-G template
C
DNA pol λ (2 nM) - + + + + + + + + - + + + + + + + +
WRN (10 nM) - - - - - + + + + - - - - - + + + +
dNTP (10 mM) - A C G T A C G T - A C G T A C G T
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Control template
8-oxo-G template
D
DNA pol λ (5 nM) - - + - + - - + - +
WRN (5 nM) - + + + + - + + + +
Lanes 1 2 3 4 5 6 7 8 9 10
K-WRN X-WRN K-WRN X-WRN
Control template
8-oxo-G template

Figure 4

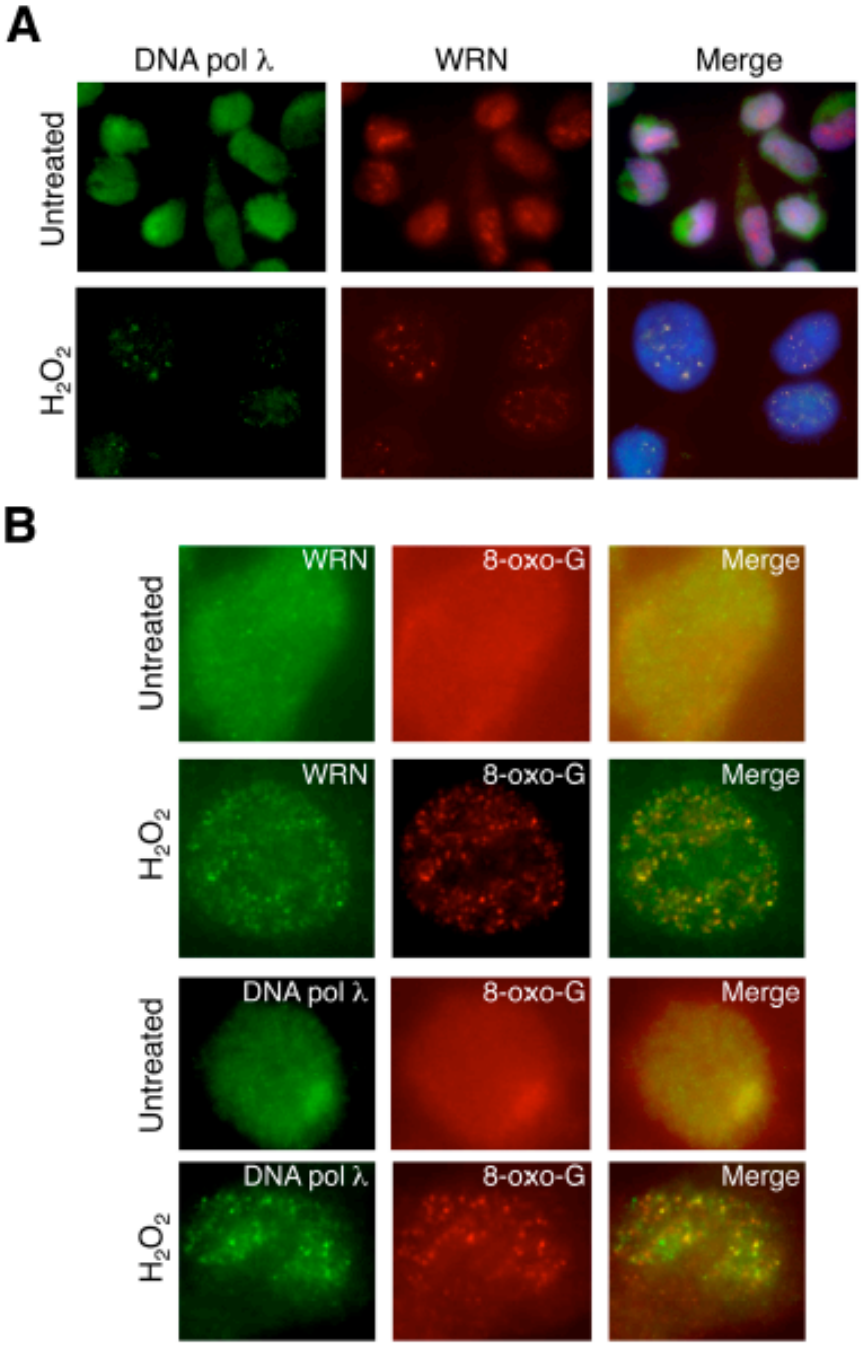
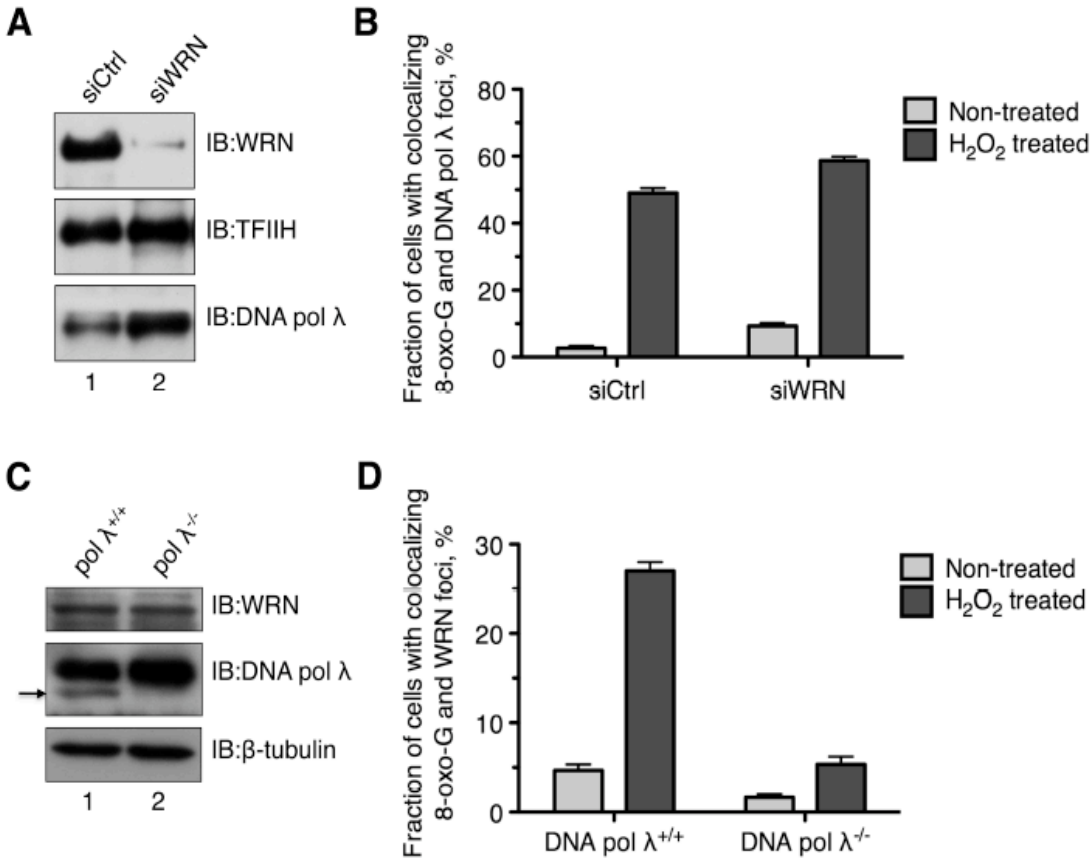
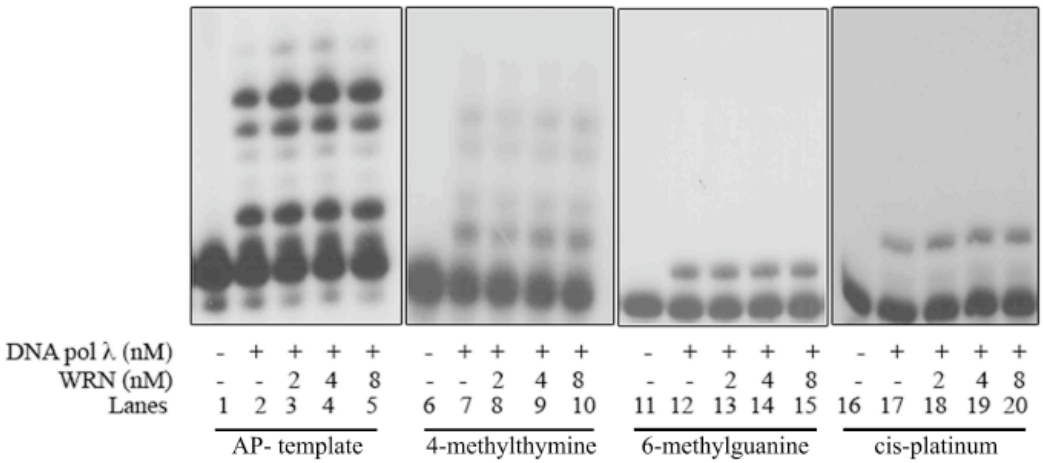

A
DNA pol λ
WRN
Merge
Untreated
H2O2
B
WRN
8-oxo-G
Merge
Untreated
H2O2
DNA pol λ
8-oxo-G
Merge
Untreated
H2O2

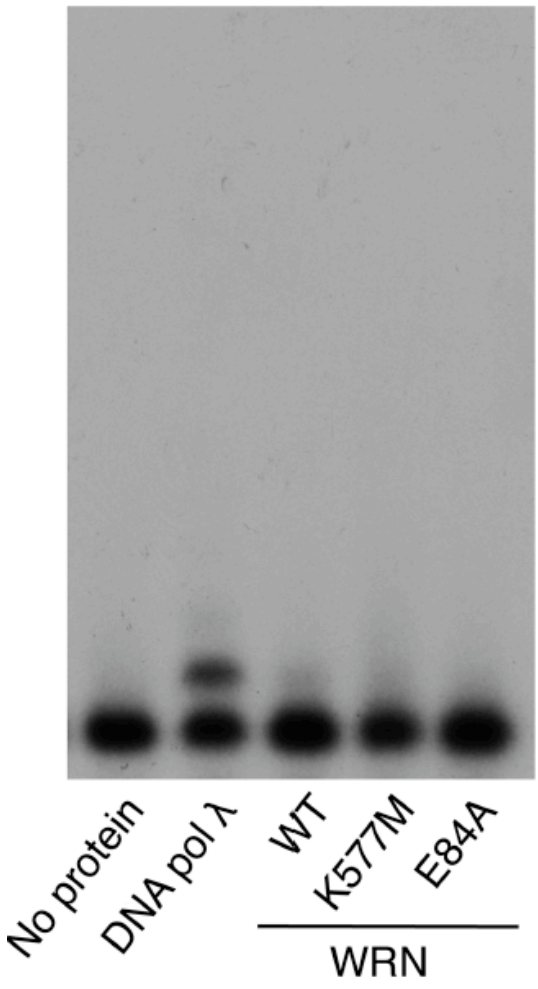
Figure 5


A
siCtrl
siWRN
IB:WRN
IB:TFIIH
IB:DNA pol λ
1
2
B
Fraction of cells with colocalizing 8-oxo-G and DNA pol λ foci, %
80
60
40
20
0
siCtrl
siWRN
Non-treated
H2O2 treated
C
pol λ+/+
pol λ-/-
IB:WRN
IB:DNA pol λ
IB:β-tubulin
1
2
D
Fraction of cells with colocalizing 8-oxo-G and WRN foci, %
30
20
10
0
DNA pol λ+/+
DNA pol λ-/-
Non-treated
H2O2 treated

FigureS1


DNA pol λ (nM) - + + + + - + + + + - + + + + - + + + +
WRN (nM) - - 2 4 8 - - 2 4 8 - - 2 4 8 - - 2 4 8
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
AP- template
4-methylthymine
6-methylguanine
cis-platinum

FigureS2



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